ASSESSMENT OF THE SENSITIZING POTENCY OF LOW MOLECULAR WEIGHT CHEMICALS

- Employment of a regression method in vivo and in vitro -

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KWANTIFICERING VAN DE MOGELIJKHEID TOT SENSIBILISATIE VAN LAAG-MOLECULAIRE VERBINDINGEN

- Toepassing van een regressiemethode met in vivo en in vitro gegevens -

(Met een samenvatting in het Nederlands)

PROEFSCHRIFT

Ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de Rector Magnificus, Prof. Dr. W.H. Gispen, ingevolge het besluit van het College voor Promoties in het openbaar te verdedigen op donderdag 7 november 2002 des middags te 12.45 uur

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CHAPTER 1

GENERAL INTRODUCTION

THE SKIN

The skin is a physical barrier between an organism and its environment. As the largest organ of the human body it prevents the loss of water from the body and protects the internal environment from outside influences. In order to understand the defensive capacities of the skin, it is important to define the structural basics of this external surface. The skin is composed of two layers: the epidermis and the dermis, and rests on the subcutaneous adipose tissue. The epidermis is a multilayered epithelium composed of several different cell types. Keratinocytes (KC) are the major cell type, and represent about 95% of the epidermal cell mass. They are responsible for the biochemical and physical integrity of the skin via their production of keratin and mucopolysaccharides (Barker *et al*., 1991). Langerhans cells (LC) comprise the second most promiment cell type in the skin. LC are bone marrow-derived dendritic cells and represent only 2% to 5% of the epidermal population. Melanocytes form some 3% of the epidermal cells and by generating the pigment melanin they protect the skin against ultraviolet radiation. Besides its barrier function, the skin has been recognized for some time as an immunologically active tissue. LC are the principal antigen-presenting cells in the skin (Katz, 1979), and KC act as signal transducers, converting non-specific exogenous stimuli into the production of cytokines, chemotactic factors and adhesion molecules (Barker *et al*., 1991).

CONTACT HYPERSENSITIVITY

Hypersensitivity reactions to drugs and industrial chemicals are relatively common in man, and often considered to be increasing at such a rate to become a major health problem in relation to environmental chemical exposures (Salvaggio, 1990; Vos *et al*., 1995). In the context of occupational and environmental health one of the most important allergic diseases caused by chemicals is contact hypersensitivity (CHS), also known as skin sensitization. CHS can occur as a result of exposure to a wide variety of chemicals and drugs (Nethercott and Holness, 1989), cosmetics (De Groot *et al*., 1988; Remaut, 1992), and various metals (Nethercott and Holness, 1989; Picardo *et al*., 1990).

CHS is not inborn, but always a consequence of earlier cutaneous contact. Individuals with CHS typically develop dermatitis (within a few days of exposure) in areas that were exposed directly to the allergen, and is characterized by perivascular inflammatory infiltration of mononuclear cells in the epidermis. Underlying the inflammation is vasodilatation resulting in redness (erythema) and increased vascular permeability with formation of oedema and induration. In this respect, the term dermatitis is used synonymously with eczema.

CHS is a rather common disease and the prevalence at any given time varies between 2-4%. Epidemiological studies comprising 20.000 individuals representing the general population showed a one year prevalence of hand eczema of 10% (Meding, 1990), 20% of which were classified as caused by contact allergy. The average duration was 12.8 years and

22% have had periods of sick leave. The risk of CHS as a result of repeated exposure to certain contact allergens (such as poison ivy) prevalent in certain areas of North and South America, may be higher than 20% (Weston and Bruckner, 2000). Furthermore, European and US studies show that children aged 0 to 14 may account for as many as 20% of cases of CHS (Barros *et al*., 1991; Goncalo *et al*., 1992; Hogan and Weston, 1993). These findings clearly show that CHS is both a common disease but also costly for society and can imply significant socio-economic consequences for the individual (Matthias, 1985).

CHS is a classical type IV delayed type hypersensitivity (DTH) reaction, caused by an abnormal response of the skin immune system (Bos and Kapsenberg, 1986) and is characterised by the infiltration of T-lymphocytes into the dermis and epidermis (Krasteva, 1993). Contact allergens are generally haptens, which are highly reactive low-molecular-weight (generally less than 500 Dalton), lipid-soluble molecules that by themselves are not recognized by the immune system. Haptens bind to proteins within the epidermis and dermis forming a complete antigen. The interaction between antigen and the T-lymphocyte of the cellular immune system is divided into two sequential phases: an initial sensitisation (induction) phase and a subsequent elicitation (challenge) phase.

Initiation phase

The sensitisation phase begins with the application of hapten to the skin surface. The hapten penetrates the epidermal barrier (stratum corneum) and binds covalently with cell surface or structural proteins, forming a hapten-carrier complex. This hapten-self component is effectively taken up by Langerhans cells (LC), specialized dendritic cells that function as the principal antigen presenting cells (APC) in the epidermis (Bergstresser *et al*., 1991). LC ingest the antigen, and process it to form small peptides before being expressed at the cell surface in association with major histocompatibility complex (MHC) molecules (Strelein and Grammer, 1989). After uptake of the antigen LC migrate via the afferent lymphatic into the lymph node (LN) where they present the antigen to the T-lymphocyte. Before LC can migrate they undergo a variety of phenotypic and morphological changes (Picut *et al*., 1987; Kolde and Knop, 1987; Aiba and Katz, 1990). Once LC, loaded with hapten-carrier complexes, arrive in the paracortical T-cell areas of the LN draining the skin, they present the antigen to CD4+ T-helper (Th) cells, which results in a clonal expansion of these cells to become antigen-specific effector T-cells. These primed T-cells migrate through the circulation to the skin or other sites and serve as a pool of (antigen-specific) memory T-cells (Knight *et al*., 1985a, 1985b).

Elicitation phase

Re-exposure of the skin to the same chemical will result in presentation of the hapten-peptide complex by Langerhans and possibly non-Langerhans antigen presenting cells to primed T-cells (Figure 1). This interaction results in activation of the antigen-specific T-cells and the release of numerous inflammatory mediators (Belsito, 1989; Bergstresser, 1990). These activated T-cells release a variety of lymphokines (cytokines) which have a variety of effects on neighbouring KC, dermal cells, blood vessels and nerve endings. CHS develops within 24 hours of antigen exposure.

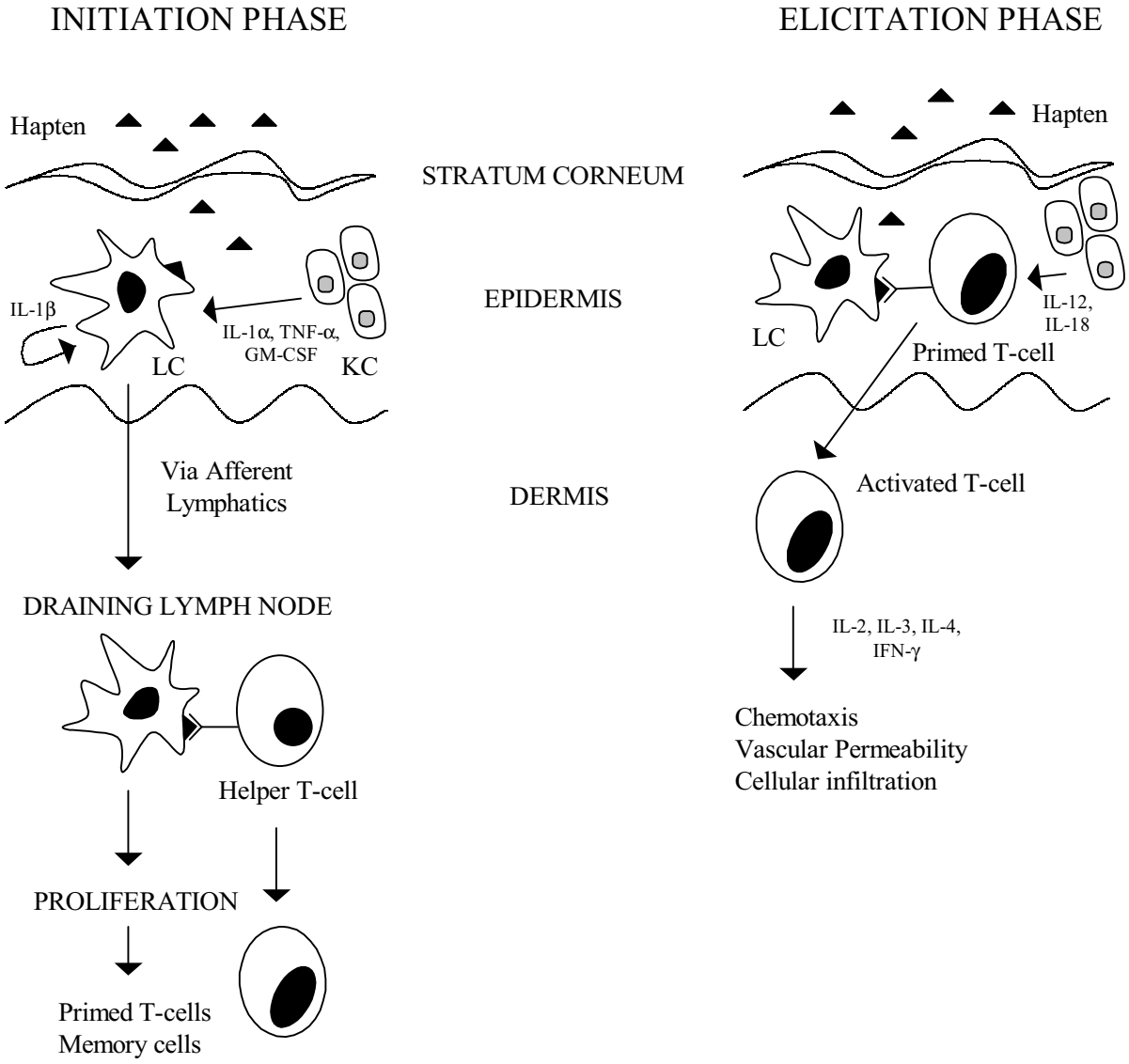

Figure 1
Induction and elicitation phase of contact hypersensitivity. Haptens penetrate the the upper epidermal layer, the stratum corneum, and bind to skin proteins. These complexes are recognized, captured, and processed by Langerhans cells (LC). After maturation, that is affected by keratinocyte (KC) –derived cytokines, hapten-bearing LC migrate to the draining lymph node were they present the hapten to T-cells. This leads to activation and the clonal expansion of T-cells and a subsequent release of these cells in the blood circulation. Upon repeated skin exposure to the hapten, skin-recruited hapten-specific T cells will cause a local cytokine-mediated inflammation.

CYTOKINE EXPRESSION IN THE SKIN

Cytokines are small polypeptide molecules that exert their biological functions in extremely low (picomolar) concentrations through specific receptors expressed on the surface of immune as well as non-immune cells (Paul, 1988; Green, 1989). They are produced locally and transiently in response to exogenous stimuli to mediate cell specific effects, including cell growth and differentiation, migration and immune functions. CHS is to a large extent regulated

by the action of cytokines. For instance, upon exposure of the skin to contact sensitizing agents, cytokine production in the epidermis by both KC and LC is immediately upregulated, thereby initiating the process of LC maturation and migration. The cytokines of greatest importance are GM-CSF, IL-1 and TNF-α (Kimber *et al*., 2000). Within the epidermis, KC are the major source of cytokines. In addition, cytokines are produced by LC and melanocytes (William and Kupper, 1996). An overview of the cytokines produced by these epidermal cells is shown in Table 1. Some of these cytokines are described in more detail in the following paragraphs.

Interleukin-1 (IL-1) was originally described as lymphocyte-activating factor and was thought to be produced only by monocytes. It is now well established, however, that many cells, including epithelial cells, endothelial cells, fibroblasts and various tumor cells, function as sources for IL-1 (Oppenheim *et al*., 1986; Kupper *et al*., 1986). Two distinct forms of IL-1, termed IL-1α and IL-1β, have been described. IL-1α was the first cytokine demonstrated to be produced by KC (Luger *et al*., 1981; Sauder *et al*., 1982). Epidermal KC constitutively synthesize IL-1α (Dinarello, 1984), and contain a large amount of preformed and biologically active IL-1α (Mizutani, 1991). Once the physical barrier of the upper epidermis is damaged, IL-1α is rapidly released so that this IL-1α reservoir represents a rapid response system for immune and inflammatory regulation in the skin, and IL-1α acts as a first line of defence against injury (Kupper, 1988). In addition, IL-1α acts as primary promoting factor in CHS reactions, stimulating further release of IL-1α and the production and release of other 'secondary' cytokines such as IL-6, IL-8 and GM-CSF (Corsini and Galli, 2000).

IL-18 is also abundantly expressed in the skin. Besides the epidermal KC and LC, IL-18 is widely expressed by both leukocytic and non-leukocytic cells including macrophages and dendritic cells. Also IL-18 is known to be constitutively produced by keratinocytes (Stoll *et al*., 1997). While recent findings have revealed that contact allergen-induced LC migration and dendritic cell accumulation in draining lymph nodes require IL-18 (Cumberbatch *et al*., 2001), it was first discovered as an interferon-γ inducing factor (IGIF) in sera of mice treated with *Myobacterium bovis* BCG and challenged with LPS (Nakamura *et al*., 1989). IL-18 acts synergistically with IL-12 to induce a Th1 response through induction of IFN-γ by T-cells (Micallef *et al*., 1996; Robinson *et al*., 1997). Also IFN-γ production by NK cells as well as the activation of NK cells are influenced by IL-18 (Hunter *et al*., 1997; Micallef *et al*., 1997). IL-18 has a direct effect on TNF-α production by T helper and NK cells (Puren *et al*., 1998). Studies in the past have revealed IL-18 as a typical Th1 cytokine. However, recent reports show that IL-18 also induces the production of Th2 cytokines from T-cells, NK cells, basophils, and mast cells (Hoshino *et al*., 1999; Yoshimoto *et al*., 1999; Nakanishi *et al*., 2001).

After activation of the skin by various stimuli, IFN-γ is the first cytokine produced by T-cells and natural killer (NK) cells (Kasahara *et al*., 1983; Howie *et al*., 1996). IFN-γ activates macrophages, neutrophils and NK cells, promoting T- and B-cell differentiation and increasing IL-1 and IL-2 synthesis. Moreover, IFN-γ increases intercellular adhesion molecule-1 (ICAM-1) and MHC II expression on epidermal KC (Bos, 1990), suggesting that KC may function as additional accesory cells in the skin able to interact with T-cells.

IL-4 plays a major role in T-cell development. It promotes differentiation of Th cells into Th2 cells during an immune response (Romagnani, 1992). Activated B-cells are induced by IL-4 to produce IgE (Coffman *et al*., 1986), and IL-4 can act as a mast cell growth factor. Mast cells and basophils produce IL-4. It has been hypothesized that IL-4 produced by these cells induces the development of Th2 cells, and that these cells in turn produce IL-4.

It has been found that IL-10, a cytokine described originally as cytokine synthesis inhibitory factor, inhibits the immunostimulatory and antigen presentation functions of LC and the ability of dendritic cells to induce IFN-γ production by T-cells (Enk *et al*., 1993; Macatonia *et al*., 1993; Peguet-Navarro *et al*., 1994; Beissert *et al*., 1995), thereby stimulating a Th2 response. IL-10 is produced by Th2 cells and keratinocytes, and is known to be upregulated in response to skin sensitization (Enk and Katz, 1992). IL-10 inhibits cutaneous inflammatory reactions (Berg *et al*., 1995), and may serve as a down-regulatory molecule for LC maturation (Kimber and Cumberbatch, 1992; Enk and Katz, 1995).

Table 1
Epidermal cytokines (after: William and Kupper, 1996; Corsini and Galli, 2000).

Cytokines	Epidermal cell types		
	Keratinocytes	Langerhans cells	Melanocytes
Interleukin 1α (IL-1α)	+	+	+
IL-1β	+	+	+
IL-3 (mouse)	+	-	-
IL-6	+	+	+
IL-7	+	-	-
IL-8 (human)	+	-	+
IL-10	+	-	+
IL-12	+	-	+
IL-15	+	+	-
IL-18	+	+	-
Granulocyte Colony-Stimulating Factor (G-CSF)	+	-	+
Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF)	+	-	+
Macrophage Colony-Stimulating Factor (M-CSF)	+	-	+
Macrophage Chemoattractant Protein 1 (MCP-1)	+	-	+
Macrophage Inflammatory Protein 1α (MIP-1α)	-	+	-
MIP-2 (mouse)	+	+	+
Interferon-inducible Protein 10 (IP-10)	+	-	-
RANTES (regulated upon activation, normal T-cells expressed and secreted)	+	-	+
Transforming growth factor α (TGF-α)	+	-	+
Transforming growth factor β (TGF-β)	+	+	+
Tumor necrosis factor α (TNF-α)	+	+	+

PRECLINICAL METHODS FOR TESTING THE CONTACT ALLERGENIC POTENTIAL OF CHEMICALS

Effective toxicologic evaluation of skin sensitization requires that potential contact allergens are identified. Much of the early work designed to investigate the phenomenon of contact sensitivity was performed in the guinea pig (Landsteiner and Jacobs, 1935) and it was this species that was chosen initially for the development of predictive tests. Since the introduction in 1944 by Draize *et al*. of the first method designed specifically for the identification of contact allergens, a variety of guinea pig methods has been described. As the guinea pig maximization test (GPMT) and the Buehler test have been much more widely used compared to any other guinea-pig assay, only these two guinea pig tests are discussed in this thesis in addition to tests in the mouse.

Guinea pig maximization test

The GPMT has been used as a method for the prediction of skin sensitizing potential for over 30 years since it was first described (Magnusson and Kligman, 1969 and 1970). During the induction phase animals are exposed intradermally (day 0), in combination with Freund's Complete Adjuvant (FCA), and topically with an occlusive patch (day 7) on the shaved scapular area. The elicitation (challenge) is performed on the shaved flank by the application of an occlusive patch. The control animals receive the vehicle during the induction phase and the tested chemical during the elicitation phase. The skin reaction is read after 24 and 48 hours as visual assessment of induced erythema and graded according to a semi-quantitative rating scale. Finally, the number of positive animals in the test group is an indication of the potency of the contact allergen. If ≥30% of the test animals scored positive, the chemical is considered a sensitizer. The GPMT is the preferred test method in the EU and >90% of the tests on new and existing chemicals were/are carried out using this method (Schlede and Eppler, 1995).

Buehler test

The Buehler test (Buehler, 1965; the technique has later been refined in 1994) is still the preferred test procedure in the USA. The main differences between the Buehler test and the GPMT are that both the induction and elicitation exposures are done topically, and no FCA is used in the Buehler test. Several experimental studies compared the Buehler test with the GPMT by testing strong sensitizers and found the Buehler test to be less sensitive than the GPMT (Marzulli and Maguire, 1982; Fahr *et al*., 1976; Frankild *et al*., 2000). Both guinea pig methods described above are recommended in the Organisation for Economic Cooperation and Development (OECD) guideline #406 (1992).

Mouse ear swelling test

More recently, different types of tests to predict the capacity of chemicals to induce contact allergy have been developed in mice. One is the mouse ear swelling test (MEST), where the activity is assessed as a function of both the degree of specific ear swelling and the percentage of test animals that exhibit a positive response (Gad *et al*., 1986). On tape-stripped abdomial skin, the test material is applied topically for 4 consecutive days in combination with FCA. Ten days following sensitization, test and control mice are challenged on one ear with the test material and on the other ear with the appropriate vehicle alone. Ear thickness is measured 24 and 48 hours later. Initially, this test was considered to be very promising but failed to prove superior to guinea pig assays. It is actually rather less sensitive, and as expensive and time-consuming as most guinea pig tests.

Local lymph node assay

An alternative approach based on the immunobiological events and responses that are provoked by contact allergens, is the murine local lymph node assay (LLNA). The LLNA is a predictive test that uses *in vivo* cell proliferation in the draining lymph nodes for assessment of the contact sensitization potential of chemicals (Kimber, 1986; Kimber and Weisenberger, 1989). Here, the uptake of [^{3}H]TdR by the local lymph node cells, as a response to the application of the test chemical to the mouse ear, is a measure for the immune response and thus is used as a measure of sensitization. A chemical proves to be positive and can be regarded a sensitizer, when it shows a threefold increase over vehicle control. In contrast to the MEST and guinea pig assays, activity is measured as a function of events occuring during the induction, rather than elicitation phase of contact sensitization.

The LLNA has been evaluated extensively for its sensitivity and reliability in predicting the contact sensitization of chemicals through both intra- and interlaboratory validation studies

(Kimber *et al*., 1991; Kimber and Basketter, 1992; Loveless *et al*., 1996) and has been found to correlate well with guinea pig studies and human analyses (Basketter *et al*., 1993; 1994).

Next, the LLNA was submitted to the Interagency Coordinating Committee on the Validation of Alternative Methods (ICVAMM, NIH 1999), for consideration as an alternative (i.e., stand-alone) test method to the guinea pig sensitization tests. It was concluded that the LLNA is an acceptable alternative to guinea pig test methods for the identification of potential contact sensitizers. Another conclusion in this evaluation was that the LLNA offers animal welfare advantages (exclusion of adjuvant, topical application instead of intradermal injection of the compound, shorter protocol) compared to the use of traditional guinea pig methods and reduces the total number of animals required.

The LLNA has been developed further to discriminate skin sensitizers from respiratory sensitizers (Dearman *et al*., 1992; Vandebriel *et al*., 2000) based on the induction of CD4+ T helper subsets. Contact allergens preferentially induce a T-helper 1 (Th1) response and mediate type IV immediate hypersensitivity, whereas respiratory allergens preferentially induce a T-helper 2 (Th2) response and mediate type I immediate hypersensitivity by the production of IgE and IgG1. These responses can be discriminated on the basis of cytokine production, such as IFN-γ, that is produced by Th1 cells, and IL-4 and IL-10, that are produced by Th2 cells. Th cells can be divided in the functional T helper subsets Th1 and Th2, according to patterns of lymphokine activity production (Abbas *et al*., 1996; O'Garra, 1998). In general, Th1 cells preferentially induce IL-2 and IFN-γ, whereas Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13 (Mossman *et al*., 1986; Openshaw *et al*., 1995). Cytokines also influence the type of T-cell subsets that are activated (Seder and Paul, 1994).

RISK ASSESSMENT IN IMMUNOTOXICOLOGY

Immunotoxicology is a relatively new area of toxicology. Immunotoxicology has grown, during the first 30 years of its existence, to a fully acknowledged subdiscipline of toxicology and may be defined as the scientific discipline concerned with the adverse effects resulting from the interaction of xenobiotics with the immune system. It includes the consequences of an action (i.e. either suppression or enhancement) by a substance (or its metabolite) on the immune system, as well as the immunological response to such a substance (IPCS, 1996; 1999).

Risk assessment has become a more structured activity during the past 50 years and is increasingly being used to form a basis for policy decisions. A fundamental goal of risk assessment is to establish a safe level of human exposure to chemicals. Unfortunately, for most compounds there is a general lack of basic toxicokinetic and mechanistic data, so that estimation of risk inevitably relies on a number of uncertainties, assumptions, and rationalisations. Initially, immunotoxicologists focused their efforts only on the identification of hazard and dose-response assessment and no attention was paid to risk assessment. Fortunately, immunotoxicologists increasingly turned to assessing the risk in relation to immunotoxic effects (Trizio *et al*., 1988; Luster *et al*., 1994; Selgrade *et al*., 1995; ILSI, 1995; Van Loveren *et al*., 1997 Descotes), and risk assessment is now emerging as a new and important field for immunotoxicology.

For contact allergy, the assessment of risk is still limited to hazard identification, and risk management is restricted to labeling. A next step in the risk assessment process should be determination of the relative potency of the substance in relation to that of other known contact allergens, which is one of the focuses in this thesis. In general, traditional test methods are not well suited for relative potency evaluation because they lack the possibility of dose-response assessment, and therefore much remains to be done for designing appropriate

quantitative experimental models useful for risk assessment of sensitization. The LLNA offers an opportunity to obtain an objective and quantitative assessment of potency as this test includes dose-response assessment. An important issue in relation to the assessment of dose-response relationships is the identification of a threshold (Kimber et al., 1999; Boukhman and Maibach, 2001). The threshold for sensitization can be defined as the highest level of exposure that fails to induce sensitization or fails to elicit an allergic reaction in a previously sensitized subject. Threshold data may provide information on safe exposure levels for humans. Exposure is determined by a combination of factors (i.e. duration, magnitude, frequency of exposure), and is usually the most neglected aspect of the risk assessment process. The final step of the process is immunotoxicity risk characterization. Unfortunately, the current lack of adequate human data is a major limitation. To overcome this difficulty, an alternative type of evaluation of the risk of adverse effects due to exposure to immunotoxic chemicals may be used; the so called parallellogram approach (Blaauboer *et al*., 1990; IPCS, 1996; Van Loveren *et al*., 1997; Figure 2). It is possible to compare the results of *in vitro* and *in vivo* animal studies using the same or similar endpoints of immunotoxicity, as it is possible to compare the results of *in vitro* studies in animals and in man. Based on the combined comparison of these results of species comparison, an improved extrapolation from animals to the human situation is now achievable. This approach may hold promise for the risk evaluation of chemicals that exert skin sensitizing properties (Van Loveren *et al*., 1998).

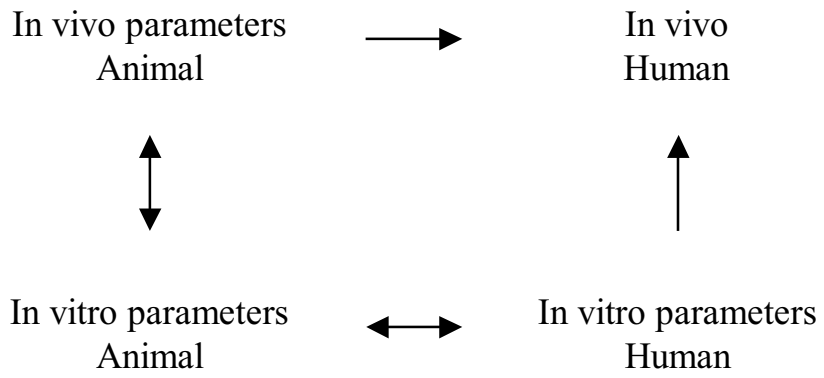

Figure 2
The parallellogram approach for immunotoxicity risk assessment.

CHEMICALS STUDIED IN THIS THESIS

Skin contact with small molecules (haptens) tends to induce cellular mediated contact sensitization. Contact sensitized individuals are at risk of developing the disease CHS if re-exposed to the specific chemical. This disease is not inborn but is always a consequence of earlier cutaneous contact, and the prevalence at any given time varies between 2-4%. It is considered to be life-long, but might become weaker when exposure is avoided. Exposure can take place in the work environment as well as at home or in leisure time and there is a considerable overlap. CHS on the hands is the most frequent example. In a survey of 564 cases of permanent disability caused by skin diseases, 222 of the 564 were caused by CHS on the hands (Menné and Bauchman, 1979). Besides the hands, CHS can also be seen in the face and ears (caused by cosmetics and medicaments), feet (after direct contact with the offending

material, most frequently chromate-tanned leather, rubber and glues; Podmore, 1995), and rest of the body as a consequense of textile dyes and formaldehyde-releasing textile resins (Fowler et al., 1992). Common high-risk occupations for CHS, common allergens associated with CHS, and the chemicals studied in this thesis, are shown in table 2, 3, and 4, respectively.

Table 2
Common high-risk occupations for CHS (Fregert, 1975).

Adhesives/plastic workers	Horticulturists
Agriculturalists	Leather tanners
Cement casters	Painters
Construction workers	Pharmaceutical/chemical workers
Glass workers	Rubber workers
Graphic workers	Textile workers
Hairdressers	Tilers
Health care workers	Wood workers

Table 3
Common allergens associated with CHS.

poison ivy, poison oak, poison sumac
other plants
nickel or other metals
antibiotics, especially those applied to the surface of the skin (topical antibiotics)
rubber and latex
cosmetics
topical anesthetics or other medications that are applied to the surface of the skin
detergents
solvents
adhesives
fragrance, perfumes
other chemicals and substances

Table 4
Compounds used in this thesis.

Allergens (alphabetical order)	Sources of exposure
Benzocaine	Topical medicament
Dibenzothiazyldisulfide (MBTS)	Production of natural rubber latex
Diethylamine (DEA)	Production of natural rubber latex
Dipentamethylenethiuramdisulfide (PTD)	Production of natural rubber latex
Dipentamethylenethiuramtetrasulfide (PTT)	Production of natural rubber latex
Dinitrochlorobenzene (DNCB)	Production of azo dyes, fungicides, rubber chemicals, and explosives
Hexylcinnamic aldehyde (HCA)	Aroma chemical
Mercaptobenzothiazole (MBT)	Production of natural rubber latex
Oxazolone	Color former
Phtalic anhydride (PA)	Production of alkyd resins and epoxy resins
Tetrabutylthiuramdisulfide (TBTD)	Production of natural rubber latex
Tetraethylthiuramdisulfide (TETD)	Production of natural rubber latex
Tetramethylthiuramdisulfide (TMTD)	Production of natural rubber latex
Tetramethylthiurammonosulfide (TMTM)	Production of natural rubber latex
Toluene diisocyanate (TDI)	Production of paints and polyurethane foams
Trimellitic anhydride (TMA)	Production of polyester
Zinc dibutyldithiocarbamate (ZDBC)	Production of natural rubber latex
Zinc diethyldithiocarbamate (ZDEC)	Production of natural rubber latex
Zinc dimethyldithiocarbamate (ZDMC)	Production of natural rubber latex
Zinc mercaptobenzothiazole (ZMBT)	Production of natural rubber latex
Zinc pentamethylenedithiocarbamate (ZPC)	Production of natural rubber latex

AIM AND DESIGN OF THIS THESIS

The scope of this thesis is to evaluate the process of risk assessment for sensitizing potency of low molecular weight chemicals, pertaining to both *in vivo* and *in vitro* approaches. The identification of sensitizing activity of chemicals is carried out on a large scale, and is performed using animal models. In general, these tests are aimed at identifying allergens. Risk assessment requires, however, besides hazard identification, also the assessment of the sensitizing potency of allergens. A major limitation to immunotoxicity risk assessment is the current lack of adequate human data.

In **chapter 2**, we used an approach based on dose-response relationships to quantify the sensitizing capacity of ten known allergens. This approach comprises non-linear regression analysis of data from a modified LLNA. Chemicals that elicit a stimulation index (SI) of 3 or more in the LLNA are considered as being sensitizers. At an SI=3, the corresponding concentrations (EC_3) were calculated and used for classification of the sensitizers based on the sensitizing potency. In addition, confidence intervals were determined to take account of the quality of the particular data set.

The standard GPMT gives a qualitative assessment rather than a quantitative evaluation as does the LLNA. To make a proper comparison between these two tests, we investigated the possibility for potency evaluation in the GPMT (**chapter 3**). With the employment of a multiple dose-response design, we evaluated the allergenic potency of three sensitizers and compared the ranking with the order of sensitizing potency seen in the LLNA. **Chapter 4** describes the method we used in chapter 2 in order to assess the allergenic potency of chemicals used in the production of latex medical gloves.

Besides hazard identification and relative potency evaluation, knowledge of the influence of exposure duration is necessary for an effective assessment of risk. We investigated if prolonged (2 months) exposure to low concentrations of allergens that do not induce an SI $\geq$ 3 in the LLNA, were able to surpass this threshold in order to investigate the influence of repeated antigen exposure (**chapter 5**).

The LLNA can also be used to discriminate skin sensitizers from respiratory sensitizers. Contact sensitizers have been shown to selectively induce Th1 responses (IFNγ) production, whereas Th2 responses (IL-4, IL10), were seen after exposure to respiratory allergens. In general, these comparisons are based on single concentrations. **Chapter 6** describes an approach that directly links the cytokine production to the proliferation of the same cells, to discriminate between respiratory and contact allergens independently of the choice of the concentrations used.

There is, as yet, no accepted *in vitro* method for the identification of skin sensitizing chemicals. In the following chapters we tried to develop approaches, without the use of laboratory animals, to evaluate the sensitizing capacity of an allergen. In the study described in **chapter 7**, we sought to estimate the sensitizing potency of allergens solely based on their physico-chemical properties. The hypothesis that the irritant capacity of allergens presents an additional risk factor, suggests that evaluating irritant potency of allergenic chemicals may be helpful to establish their allergenic potency. Epidermal keratinocytes are known to produce and excrete a wide range of cytokines. The anatomical location of keratinocytes and their significant role in the development of CHS justifies the use of keratinocyte-derived cytokine production as read-out to evaluate sensitizing potency. In **chapter 8** we determined the sensitizing potency of chemicals as a function of keratinocyte cytokine expression. Cytokine dose-response data were evaluated in a murine keratinocyte cell line by non-linear regression analysis and at a stimulatory index (SI) of three the corresponding estimated concentration was calculated (EC_3). We used these EC_3 values to rank these chemicals and to compare the

outcome of the present study with those obtained from the local lymph node assay (LLNA). **Chapter 9** extends the latter study by evaluating cytokine dose-response data in a human keratinocyte cell line. These data may tell us whether the ranking based on the cytokine responses in the human keratinocyte cell line is in accordance with the ranking derived in the mouse (both *in vitro* as *in vivo*). In addition, it provides information about the differences in sensitivity between the different species (man vs mouse). Based on the combined comparison of these results, an improved extrapolation from animals to humans may be achieved. Finally, conclusions of the findings in the preceding chapters are summerized and discussed in **chapter 10**.

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CHAPTER 2

A QUANTITATIVE METHOD FOR ASSESSING THE SENSITIZING POTENCY OF LOW MOLECULAR WEIGHT CHEMICALS USING A LOCAL LYMPH NODE ASSAY: EMPLOYMENT OF A REGRESSION METHOD THAT INCLUDES DETERMINATION OF THE UNCERTAINTY MARGINS

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Risk assessment of sensitizing chemicals requires besides hazard identification, the assessment of potency. To examine the sensitizing capacity of low molecular weight chemicals, a murine local lymph node assay (LLNA) was used. The sensitizing capacity of known allergens was quantified by dose-response modeling. At a stimulatory index (SI) of three, the corresponding estimated concentration was calculated (EC_3), together with a confidence interval to take account the quality of the particular data set. We tested ten allergens (ethyl-p-aminobenzoate (benzocaine), diethylamine (DEA), 2,4-dinitrochlorobenzene (DNCB), 2- mercaptobenzothiazole (MBT), 4-ethoxymethylene 2-phenyloxazol-5-one (oxazolone), phthalic anhydride (PA), toluene diisocyanate (TDI), trimellitic anhydride (TMA), tetramethyl thiuram disulfide (TMTD) and zinc dimethyl dithiocarbamate (ZDMC)). Oxazolone showed the strongest sensitizing potency followed in this order by DNCB, TDI, TMA, PA, TMTD, ZDMC, MBT, benzocaine and DEA. The approach performed in this study is a way to accurately assess the potency of sensitizing chemicals and thus a possibility for classification.

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INTRODUCTION

Methods for the identification of sensitization hazards have been available for many years. The tests most commonly used to identify skin sensitizing capacity are the guinea pig maximization test (GPMT) using adjuvant (Magnusson and Kligman, 1969) and the occluded patch test of Buehler without adjuvant (Buehler, 1965) in the guinea pig. More recently, the murine local lymph node assay (LLNA) (Kimber et. al., 1986; Kimber and Weisenberger, 1989; Kimber and Basketter, 1992) was introduced and validated for various chemicals. The results obtained in these tests give the possibility for labeling and classification of sensitizing chemicals.

The GPMT has been used as the preferred method for predicting skin sensitization for over 25 years since it was first described. Although the GPMT is able not only to detect chemicals with moderate and strong sensitizing potential but also chemicals with relatively weak sensitizing potential (Robinson, 1990), it has its drawbacks compared to the LLNA. In contrast to the GPMT the LLNA is able to detect allergic potency based on a quantitative endpoint instead of visual (semi-quantitative) assessment of challenge induced erythema. Moreover, the GPMT has disadvantages including the use of an adjuvant and the length and complexity of the test.

The LLNA is a method for the predictive identification of chemicals that have the potential to cause sensitization. In the LLNA assessment of immune reactivity of an immune (allergy) response is determined in the induction phase. The uptake of [^{3}H]TdR by the local lymph node cells, as a response to the application of the test chemical to the mouse ear, is a measure for the immune response and thus can be used as a measure of sensitization.

Lymph node proliferative responses of treated animals are compared to those of non-treated or vehicle-treated animals. Chemicals that elicit a stimulation index (SI) of 3 or more in the LLNA are considered as being sensitizers. Currently, these EC_3 (estimated concentration in % required for SI=3) values are used for the comparison of sensitizing potential derived from local lymph node responses (Kimber *et al*., 1995; Kimber and Basketter, 1997; Loveless *et al*., 1996). In the GPMT the potency is based on the percentage of positive animals using a single dose. However, for determination of the potency based on lowest effective dose levels, dose-response studies are required. In the GPMT both for the induction and challenge phase dose-response studies would be needed. As the LLNA gives a more quantitative result, we feel that the LLNA is more suited for estimation of lowest effective doses in a dose-response study. To increase the sensitivity of the LLNA for weak allergens all animals were pretreated with sodium dodecyl sulphate (SDS).

In this study the known sensitizers ethyl-p-aminobenzoate (benzocaine), diethylamine (DEA), 2,4- dinitrochlorobenzene (DNCB), 2- mercaptobenzothiazole (MBT), 4-ethoxymethylene 2- phenyloxazol-5-one (oxazolone), phthalic anhydride (PA), toluene diisocyanate (TDI), trimellitic anhydride (TMA), tetramethyl thiuram disulfide (TMTD) and zinc dimethyl dithiocarbamate (ZDMC) were evaluated.

METHODS

Animals

Young adult (6-8 weeks old) male/female BALB/c strain mice were used for the experiments. They were obtained from our own breeding colony. The animals were bred specific pathogen free and kept under conventional conditions. The mice were fed Hope Farms chow pellets (Woerden, NL) and water *ad libitum*.

Chemicals

Benzocaine (ethyl-p-aminobenzoate; 99% purity; Sigma-Aldrich Chemie B.V., Zwijndrecht NL), DEA (diethylamine; 99.5%; free base solution; Sigma-Aldrich), DNCB (2,4-dinitrochlorobenzene; 98%; Sigma-Aldrich), MBT (2-mercaptobenzothiazole; 98%; Sigma-Aldrich), oxazolone (4-ethoxymethylene 2- phenyloxazol-5-one; 90%; Sigma-Aldrich), PA (phthalic anhydride; 99%; Sigma-Aldrich), TDI (toluene 2,4-diisocyanate; 99.8%; Sigma-Aldrich), TMA (trimellitic anhydride; 1, 2, 4- benzenetricarboxylic anhydride; 97%; Sigma-Aldrich), TMTD (tetramethylthiuramdisulfide; 98%; Sigma-Aldrich) and ZDMC (zincdimethyldithiocarbamate; 90%; Fluka, Zwijndrecht NL) were tested in 4:1 acetone/olive oil (AOO). SDS (sodium dodecyl sulphate; >99%; Merck B.V., Amsterdam, NL) was dissolved in 4:1 acetone/olive oil (AOO).

Table 1
Concentrations used in the local lymph node assay (LLNA)

Chemical	% Concentration	n
Benzocaine	7.5, 15, 22.5, 30	6
DEA	2.5, 5, 10, 20, 40	3
DNCB	0.05, 0.1, 0.25, 0.5, 1	3
MBT	0.1, 1, 5, 10, 17.5	4
Oxazolone	0.0004, 0.0012, 0.0037, 0.011, 0.033, 0.1	3
PA	0.25, 1, 2.5, 10, 25	3
TDI	0.25, 0.5, 1, 2.5, 5	3
TMA	2.5, 5, 10, 25, 50	3
TMTD	0.0312, 0.0625, 0.125, 0.25, 0.5, 1	3
ZDMC	0.375, 0.75, 1.5, 3, 6	3

n= number of animals (per concentration)

Local lymph node assay (LLNA)

Groups of mice (n= 3,4, or 6) were pretreated with 1% SDS (w/v) one hour before exposing the animals to 25 µl of test solution in vehicle or vehicle alone on both ears daily for three consecutive days. A positive response is not seen at the SDS concentration that we used (1%). However, application of 1% SDS and the test chemical generally resulted in an increased response compared to the test chemical alone (data not shown).

The concentrations of the test chemicals used are presented in Table 1. Three days following the last topical application, the auricular lymph nodes were excised. The lymph nodes were weighed and pooled for each animal and suspended in 5 ml RPMI- 1640 (Gibco, Breda, NL) supplemented with 5% heat inactivated Fetal Calf Serum (PAA, Linz, Austria), 100 U/ml penicillin and 100 µg/ml streptomycin (standard medium). Single cell suspensions were prepared under aseptic conditions by pressing the lymph node through a sterile 70 µm nylon cell strainer (Falcon, Franklin Lakes, USA). The cells were washed twice in standard medium (10 minutes, 311g, 4°C) and resuspended in 1 ml standard medium with 10% FCS. The cells were counted using a Coulter Counter (Coulter Electronics, Mijdrecht, NL) and cultured at a concentration of 1.10^7 cells/ml. When necessary, cell suspensions of several animals were pooled to obtain the concentration required. The cell suspensions (200 µl) were seeded in triplicate into round-bottomed 96-well microtitre plates (Greiner, Alphen a/d Rijn, NL). The cells were cultured with 10 µl of [^{3}H]TdR (Amersham, Buckinghamshire, UK; 37 kBq/ml) for 24 hr at 37°C in a humidified atmosphere of 5% CO_2 in air. The [^{3}H]TdR incorporation was determined by liquid scintillation counting in a β plate counter (1205 Betaplate^{TM} Wallac, Turku, Finland). The [^{3}H]TdR incorporation is expressed per animal, i.e.

the [^{3}H]TdR incorporation is multiplied by the cell number of the two lymph nodes and devided by the cell number in culture.

To study the potency of sensitizers, we have used a range of test concentrations to determine dose-response relationships on which the quantitative estimation of the allergic potency was based. The estimated concentration in % required for SI=3 (EC_3) was determined as the estimated dose inducing a stimulation index of three between treated versus control.

Statistical analysis

The dose-response data were analysed by nonlinear regression analysis, using the following family of models:

model 1: $y = a$

model 2: $y = a \exp(b x)$

model 3: $y = a \exp(b x^d))$

model 4: $y = a (c - (c - 1) \exp(b x)$

model 5: $y = a (c - (c - 1) \exp(b x^d))$.

where y represents the response ([^{3}H]TdR incorporation) and x the applied concentration.

In these models the parameter *a* represents the background [^{3}H]TdR incorporation of the particular assay. The parameter *b* reflects the 'slope' or the 'strength' of the response with increasing dose. The selection of the model to be used for a particular data set follows from a procedure of successively fitting the above models, and applying likelihood ratio tests to establish if an increase in the number of parameters leads to a significantly better fit to the data. A model with more parameters is considered better only if this leads to a significantly better fit (Slob, 1999). Then the selected model is used to derive the concentration (EC_3) associated with a stimulation index of 3. The uncertainty in the estimate of the EC_3 is assessed by a bootstrap method (Slob and Pieters, 1998), resulting in an uncertainty distribution from which any desired confidence interval can be derived. In this paper the 5% and 95% confidence limits are reported (i.e. 90%-confidence intervals).

RESULTS

The results obtained with the various chemicals are shown in Figure 1. The left panels show the [^{3}H]TdR incorporation as a function of the concentration with the fitted regression function, and the estimated concentration at a stimulation index of three (EC_3) in the [^{3}H]TdR incorporation per animal. The uncertainty distributions for the EC_3 values are shown in the right panels. The dose-response data were analysed by nonlinear regression analysis. Based on the criteria, namely a threefold increase over vehicle control, every compound tested proved to be positive and can therefore be regarded as a sensitizer. Differences in potency were observed between different chemicals. An exponentially shaped curve was seen for six of the chemicals, three of the chemicals showed a sigmoïdally shaped curve and one curve showed a logarithmic dose-response profile. These differences in the shapes of the dose-response curves result from the selection of the model to be used for the particular data set, as discussed in Section 2.4.

Lymph node weights and cell counts are presented in Table 2. Based on these data DNCB, oxazolone, PA, TDI and TMA are considered as being sensitizers according to the criteria for a positive response. ZDMC is regarded as a sensitizer based on the cell counts alone. In general, the data in Table 2 shows a ranking quite similar to the data shown in Figure 1 and presented in Table 3. However, lymph node proliferation instead of lymph node weights and cell counts seems to be a better indicator of the sensitizing potential of a chemical, as was first suggested by Kimber and Dearman (1991).

Table 2
Lymph node weights and cell counts after epicutaneous treatment of BALB/c mice.

Chemical	% Concentration	Lymph node weight/animal (mean ± SD, in mg)	Cell counts/animal (mean ± SD x 10^6)
Benzocaine	0	5.20 ± 1.31	4.91 ± 1.12
	7.5	9.22 ± 1.75	6.83 ± 2.50
	15	6.87 ± 1.24	11.04 ± 2.71
	22.5	8.67 ± 0.60	9.77 ± 2.00
	30	8.69 ± 0.92	11.07 ± 1.59
DEA	0	2.42 ± 0.49	3.09 ± 0.26
	2.5	2.63 ± 0.20	3.20 ± 0.56
	5	2.65 ± 0.23	3.18 ± 0.05
	10	2.82 ± 0.19	3.87 ± 0.91
	20	2.87 ± 0.30	4.12 ± 0.48
	40	4.20 ± 0.57	7.54 ± 1.64
DNCB	0	2.23 ± 0.54	4.40 ± 1.11
	0.05	3.21 ± 0.50	7.52 ± 1.54
	0.1	4.95 ± 0.63	11.69 ± 0.34
	0.25	9.87 ± 1.39	23.00 ± 3.22
	0.5	10.15 ± 0.88	21.20 ± 5.10
	1	13.93 ± 1.31	29.60 ± 5.54
MBT	0	2.91 ± 0.41	4.22 ± 1.10
	0.1	2.93 ± 0.20	4.62 ± 1.13
	1	2.95 ± 0.49	5.07 ± 1.17
	5	3.70 ± 0.44	4.78 ± 1.00
	10	4.00 ± 0.42	5.61 ± 0.83
	17.5	4.24 ± 0.91	4.80 ± 0.71
Oxazolone	0	2.42 ± 0.49	3.09 ± 0.26
	0.0004	2.52 ± 0.56	3.28 ± 0.91
	0.0012	2.80 ± 0.21	3.69 ± 0.43
	0.0037	3.68 ± 0.79	5.33 ± 0.15
	0.011	4.60 ± 0.32	6.35 ± 1.51
	0.033	6.08 ± 0.83	10.30 ± 2.52
	0.1	11.40 ± 1.22	21.40 ± 2.51
PA	0	2.53 ± 0.46	5.42 ± 0.91
	0.25	4.10 ± 0.58	9.53 ± 2.00
	1	5.83 ± 0.90	13.19 ± 3.61
	2.5	6.12 ± 1.55	16.28 ± 4.12
	10	10.77 ± 1.43	25.01 ± 8.36
	25	11.10 ± 2.56	29.44 ± 2.10
TDI	0	2.75 ± 0.58	4.60 ± 0.50
	0.25	5.17 ± 1.31	12.78 ± 4.30
	0.5	9.78 ± 1.79	21.72 ± 6.79
	1	13.57 ± 2.36	33.20 ± 7.24
	2.5	13.13 ± 2.52	26.71 ± 6.58
	5	14.30 ± 3.06	25.57 ± 9.22
TMA	0	2.23 ± 0.54	4.40 ± 1.11
	2.5	7.78 ± 1.16	15.29 ± 1.60
	5	9.68 ± 1.14	22.00 ± 0.54
	10	12.37 ± 1.07	31.04 ± 5.22
	25	11.20 ± 1.11	23.70 ± 6.64
	50	10.73 ± 1.16	27.91 ± 2.57

Chemical	% Concentration	Lymph node weight/animal (mean ± SD, in mg)	Cell counts/animal (mean ± SD x 10^6)
TMTD	0	2.87 ± 0.20	3.43 ± 0.25
	0.0312	3.38 ± 0.31	4.43 ± 1.61
	0.0625	2.70 ± 0.49	3.38 ± 0.74
	0.125	3.15 ± 0.42	4.25 ± 0.45
	0.25	3.27 ± 0.26	4.36 ± 1.41
	0.5	3.95 ± 0.58	6.23 ± 1.08
	1	4.75 ± 0.81	7.06 ± 2.30
ZDMC	0	2.87 ± 0.20	3.43 ± 0.25
	0.375	3.28 ± 0.35	4.94 ± 1.76
	0.75	3.47 ± 0.51	5.00 ± 2.03
	1.5	4.78 ± 0.76	7.61 ± 1.32
	3	5.35 ± 1.45	10.50 ± 3.48
	6	8.02 ± 2.23	16.00 ± 5.20

The EC_3 values of [^{3}H]TdR incorporation per animal and the associated confidence intervals derived from the experiments are summarized in Table 3. The chemicals are ranked according to their EC_3 values. The classification formerly obtained with the GPMT, and the LLNA as performed by other groups, and information on whether the test compounds are known sensitizers in humans are also presented in Table 3. The ranking according to the estimated EC_3 values presented in Table 3 is quite similar to the classification derived from data formerly obtained in the GPMT and the LLNA.

DISCUSSION

The LLNA is used as a test for predicting sensitization in humans. A chemical that induces an SI of three or more is regarded as a sensitizer. Recently, it has been suggested that simple linear interpolation between the observed responses on either side of the threefold stimulation index provides a robust assessment of the EC_3, without the need for recourse to more sophisticated statistical techniques (Basketter et. al., 1999). Rather than using the SI of three as a cut-off point or limit, our method evaluates all data points (animals) contributing to the dose-response curve to determine the EC_3. In addition, information on the reliability of the data is obtained using the bootstrap approach, providing confidence limits. This renders the potency assessment more reliable. Also for very weak sensitizers inducing responses below an SI of three, the concentration producing an SI of three can be estimated based on the curve fitting method, together with its confidence limits. In such a case very high, and possibly unrealistic, sensitizing concentrations may be obtained.

It has been reported that the LLNA can detect contact allergens defined as moderate, strong or extreme, but not those defined as mild or weak (Basketter and Scholes, 1992). In the present study all chemicals reached an SI of three within the observed dose ranges and were thus identified as sensitizers. This may be due to the fact that in order to increase the sensitivity for weak allergens the animals were pretreated with SDS. This chemical is an example of an irritant that can give a positive response in the LLNA (Gerberick *et al*., 1992; Montelius *et al*., 1994). The pretreatment with SDS was done to increase the sensitivity of the LLNA, aiming at obtaining positive results for weak allergens. Pretreatment with SDS combined with application of the chemical showed an increased reponse compared to treatment with the chemical alone. In addition, the application of 1% SDS only gave no stimulation in the LLNA (data not shown).

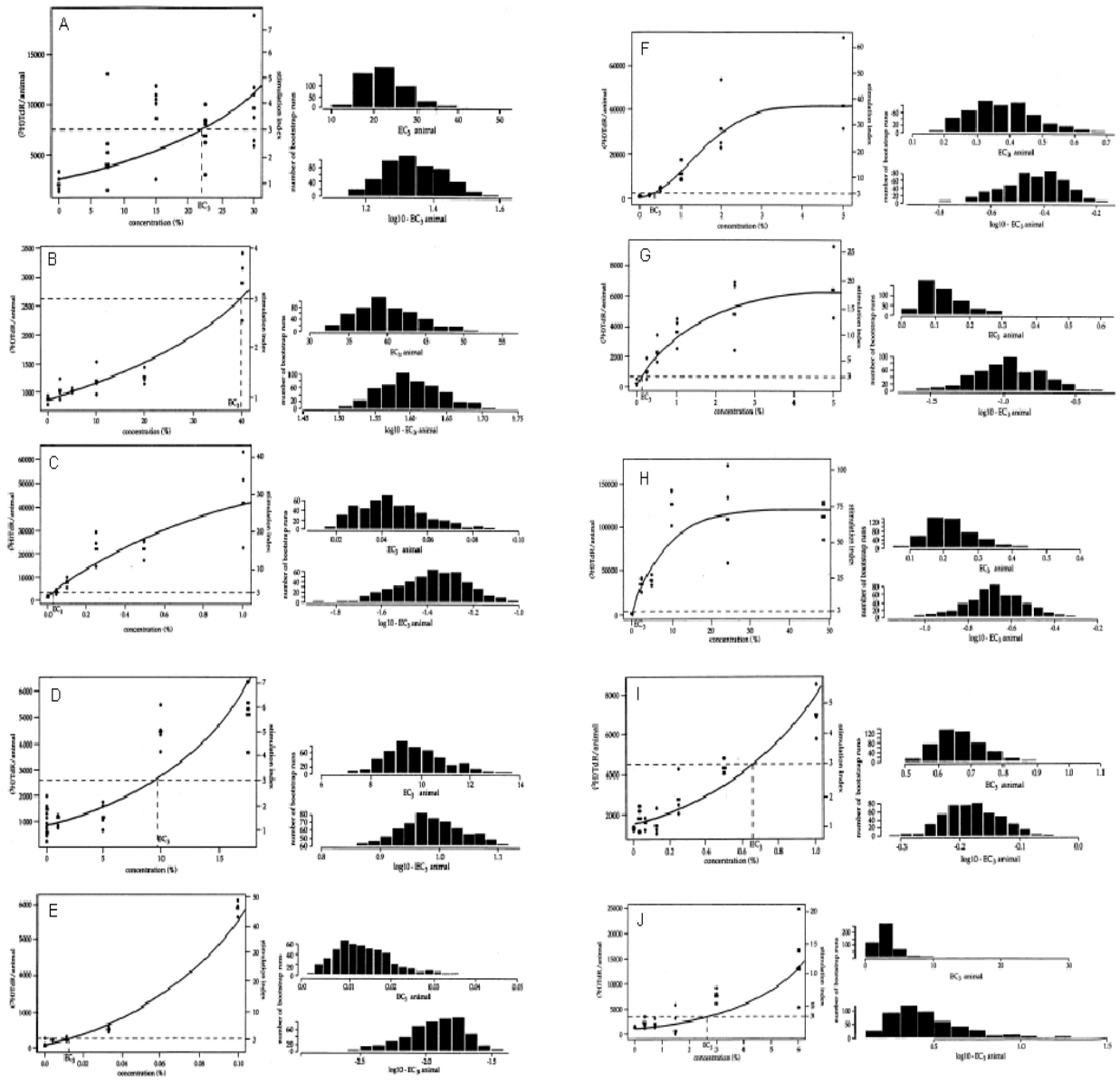

Figure 1
Local lymph node dose response curves. Left panels: [^{3}H]TdR incorporation (cpm per animal) of the local lymph node cells as a function of concentration (dots refer to individual animals; lines refer to averages per concentration), with fitted regression function at a stimulation index of three and the estimated EC_3 values (in %). The models used for fitting were model 2 for benzocaine (A), DEA (B), MBT (D), TMTD (I) and ZDMC (J); model 3 for oxazolone (E); model 4 for DNCB (C), TDI (G) and TMA (H); model 5 for PA (F). Right panels: the associated uncertainty distribution (obtained with 500 bootstrap runs from the fitted regression function) for the EC_3 values, shown on a linear scale (upper graph) and a log-scale (lower graph).

Oxazolone was the most potent sensitizer in this study with an estimated EC_3 of 0.013% and an uncertainty range of 0.004-0.025%. This is approximately one order of magnitude higher than previous findings in the LLNA with predicted values ranging from

0.0007 to 0.0026% (Loveless *et al*., 1996). DNCB, known to cause allergic contact dermatitis in man (Kligman and Epstein, 1959), showed an estimated EC_3 of 0.044%, consistent with earlier findings obtained in several laboratories (Kimber *et al*., 1995; Loveless *et al*., 1996). Also TDI, TMA, PA and TMTD showed an estimated EC_3 below 1%. PA and TMA can penetrate the skin of humans and thus have the potential to elicit an allergic reaction of the skin (Bernstein *et al*., 1982). The contact sensitivity of TDI has also been demonstrated by some epidemiological investigations and animal experiments (Malten, 1979; Tominaga *et al*., 1985). TMTD is regarded as a moderate sensitizer based on the results obtained in the GPMT (Magnusson and Kligman, 1969; 1970). This was also found for ZDMC (Matsushita *et al*., 1977; 1978). Besides, it has been reported that ZDMC as well as DEA, MBT and TMTD are able to cause allergic contact dermatitis by rubber gloves in humans (Kaniwa *et al*., 1993). In summary, all the chemicals tested in this paper have proven their sensitizing potency based on experimental and/or clinical data.

Table 3
The EC_3 values and uncertainty distribution of the chemicals tested, classification of chemicals in the local lymph node assay (LLNA) and the guinea pig maximization test (GPMT), and information on sensitizing capacity in humans.

			Classification of sensitizing potential[1]		
Chemical	EC_3 (%)	L05-L95*	GPMT	LLNA	Human
Oxazolone	0.013	0.004-0.025	Extreme[2]	+[3]	NA
DNCB	0.044	0.025-0.078	Extreme	+	Yes[10]
TDI	0.109	0.048-0.263	NA	+[8]	Yes[11]
TMA	0.218	0.128-0.405	Moderate	+	Yes[12]
PA	0.357	0.226-0.560	Extreme	+	Yes[12]
TMTD	0.659	0.554-0.815	Moderate[4]	±[6]	Yes[13]
ZDMC	2.670	1.631-8.326	Moderate[5]	NA	Yes[13]
MBT	9.669	8.020-12.189	Moderate[4]	±[6]	Yes[13]
Benzocaine	22.026	16.576-33.953	Mild[4]/ Moderate	−[1]/±[9]	Yes[14]
DEA	39.784	34.078-47.703	NA	NA	Yes[13]

*; 5th and 95th percentile
1; Basketter and Scholes (1992), except where indicated
2; Gad et al. (1986)
3; Loveless et al. (1996)
4; Magnusson and Kligman (1969, 1970)
5; Matsushita et al. (1977, 1978)
6; Ikarashi et al. (1993)
8; Dearman et al. (1996), (SI=12 for TDI 0.75%)
9; Basketter et al. (1995)
10; Kligman and Epstein (1959)
11; Malten (1979), Tominaga et al. (1985)
12; Bernstein et al. (1982)
13; Kaniwa et al. (1993)
14; Cronin (1980)
NA; data not available
+; strong, ±; moderate, −; weak
EC_3; estimated concentration in % required for SI=3

The magnitude of the effect in the animal is estimated from the dose-response data obtained from the LLNA. The endpoint of the LLNA is proliferation per animal measured by [^{3}H]TdR incorporation and the criteria for a positive response is a threefold increase in proliferation over vehicle control. The precision of the estimated EC_3 is mainly governed by the total

number of animals in the study. In most cases, three animals per group and five concentrations were used. Testing more concentrations benefits the benchmark approach (Slob and Pieters, 1997), making the results more reliable. More reliable results can be obtained by increasing the number of concentrations investigated and, compared to conventional toxicology, reducing the number of animals per group. However, a minimum of 3 animals per group seems warranted in order to exclude individual extreme (outlier) responses.

The chemicals tested can now be ranked based on the results presented in this paper. The ranking of the chemicals shown in Table 3 is quite similar to the ranking according to their sensitizing potential in the GPMT, and LLNA established by other groups. An exception is the relative potency of TMA and PA; the former is classified as more potent according to our approach, whereas the GPMT identifies PA as the more potent sensitizer (Basketter and Scholes, 1992). Benzocaine and TMTD are both known as moderate sensitizers in the classification of sensitizing potential performed in the GPMT and the LLNA (Table 3). However, according to the data presented in this paper EC_3 values showed a considerable (30-fold) difference between these two sensitizers. So, a more accurate distinction can be made between benzocaine and TMTD in terms of classification as sensitizers. In addition, the observed differences in EC_3 values also have an impact when doing risk assessment for these compounds. The quality of the data is translated to uncertainty margins which benefits the comparison between the sensitizers. For reasons of safety it would be better to make use of the calculated 5% confidence limit instead of the point estimate of the EC_3. The lower 5% confidence limit, predicts that below that particular dose there still is a 5% chance for a positive response in the test with an SI of three. It should be kept in mind that even when sensitizers are classified as strong inducers of allergy, the actual risk for developing allergy is also determined by exposure.

In conclusion, the sensitizing potential of ten chemicals was evaluated using a dose-response analysis for LLNA data. The sensitization ability was expressed as the concentration able to elicit a stimulation index of three compared to the vehicle control. The sensitizing potential of oxazolone was strongest, followed by DNCB, TDI, TMA, PA, TMTD, ZDMC, MBT, benzocaine and DEA. The approach performed in this study is a way to accurately assess the potency of sensitizing chemicals. This enables the estimation of the lowest concentration needed for sensitization, and to use these data for risk evaluation.

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CHAPTER 3

COMPARISON OF DOSE-RESPONSES OF CONTACT ALLERGENS USING THE GUINEA PIG MAXIMIZATION TEST AND THE LOCAL LYMPH NODE ASSAY

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The guinea pig maximization test (GPMT) has been used as a method for the prediction of skin sensitizing potential for over 30 years. Besides hazard identification, risk assessment of sensitizing chemicals requires the assessment of potency. For the determination of potency based on lowest effective dose levels, dose-response studies are required. In the standard GPMT a single concentration is used for intracutaneous and topical induction and the assay provides a qualitative assessment of allergenicity. This paper presents data derived from quantitative evaluation of the sensitizing potency of chemicals in the GPMT, based on multiple concentrations. We performed the GPMT in accordance with the original procedure of Magnusson and Kligman; and included in this procedure a range of intradermal and topical concentrations for induction. Three allergens with different sensitizing potencies, diethylamine (DEA), tetramethyl thiuram disulfide (TMTD) and zinc dimethyl dithiocarbamate (ZDMC) were tested. The data obtained with this test procedure were compared to data we previously obtained using the local lymph node assay (LLNA). Both the GPMT and the LLNA showed dose response relationships for the three chemicals tested. For the chemicals tested, both tests differed in the relative potencies based on benchmark concentrations. While both tests ranked DEA as the least potent allergen, the GPMT ranked ZDMC more potent than TMTD, the reverse being found in the LLNA. The nature of the data provided in the LLNA makes it likely that benchmarks as defined with this test are more reliable than that defined in the GPMT. However, further validation with human data is necessary.

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INTRODUCTION

The guinea pig maximization test (GPMT), that includes Freund's Complete Adjuvant (Magnusson and Kligman, 1969), and the occluded patch test of Buehler, that does not use adjuvant (Buehler, 1965), are the tests most commonly used to identify skin sensitizing capacity. The results obtained in these tests give the possibility for labeling sensitizing chemicals. However, risk assessment of sensitizing chemicals requires besides hazard identification, the assessment of potency. For the determination of potency based on lowest effective dose levels, dose-response studies are required. In the standard GPMT and Buehler test a single concentration is used for intracutaneous and/or topical induction and thus gives a qualitative assessment rather than a quantitative evaluation of allergenicity.

An alternative test for assessing skin sensitization potential is the local lymph node assay (LLNA) (Kimber et. al., 1986; Kimber and Weisenberger, 1989; Kimber and Basketter, 1992). The LLNA is a method in which the measurement takes place in the induction phase of contact hypersensitivity, rather than the elicitation phase. Besides the advantage of improved animal welfare (exclusion of adjuvant, topical application instead of intradermal injection of the compound, shorter protocol) the LLNA is, compared to the GPMT, also able to detect allergenic potency based on a quantitative endpoint instead of visual (semi-quantitative) assessment of challenge induced erythema. The LLNA identifies sensitizing chemicals, and has been subject to a validation study (ICVAMM, US Interagency Coordinating Committee on the Validation of Alternative Methods, NIH 1999), and on the basis of the outcome will be used as a stand-alone test. A next step, besides the identification, is to establish the sensitizing potency of allergens. Relative potency determination with the LLNA has been descibed in previous papers (Basketter *et al*., 2000; Van Och *et al*., 2000).

In the standard GPMT, a single concentration is used for intracutaneous and/or topical induction and thus gives a qualitative assessment rather than a quantitative evaluation of allergenicity. To establish the possibility for potency evaluation with the GPMT, we chose to make a comparison between the LLNA and the GPMT in this respect. We therefore performed dose response studies in both the LLNA and the GPMT. In this paper, we were especially focused on the assessment of potency of the different chemicals rather than the identification of sensitizers in general.

In the present study we use a multiple dose-response design for the GPMT, to evaluate the allergenic potency derived in the elicitation phase of contact hypersensitivity. The sensitizers diethylamine (DEA), tetramethyl thiuram disulfide (TMTD) and zinc dimethyl dithiocarbamate (ZDMC) were evaluated in this study. They are used as accelerators in the manufacturing of natural rubber latex products such as medical gloves. All three chemicals are able to induce allergic contact dermatitis in humans by use of rubber gloves (Kaniwa *et al*., 1993). TMTD has been classified as a moderate sensitizer based on the results obtained in the GPMT (Magnusson and Kligman, 1969; 1970) and the LLNA (Ikarashi *et al*., 1993). ZDMC has been evaluated using the GPMT only and was also identified as a moderate allergen (Matsushita *et al*., 1977; 1978). Using the LLNA, we recently identified TMTD as the strongest sensitizer followed in this order by ZDMC and DEA (Van Och *et al*., 2000). Based on these latter results, TMTD and ZDMC were classified as moderate sensitizers, whereas DEA was identified as a weak allergen.

These three compounds were studied in the GPMT. In the present study both the intradermal and the topical induction were applied in an increasing concentration. This multiple-dose-response induction protocol provides dose-response relationships that are essential for risk assessment of contact allergens. Dose response studies using the GPMT have been described by others (Nakamura *et al*., 1994; Andersen *et al*., 1995). As read-out of the

test, we took into account not only the percentage of reacting animals (as is usually done), but also the severity of the responses of the skin, measured at 24 or 48 hours. The maximal skin scores for each animal were summed per dose group, resulting in a total skin score. The potency of the sensitizing capacity, based on the effective concentrations (i.e. those causing minimal detectable skin reactions in the adapted GPMT), were compared to the potency as derived in the modified LLNA, performed in a previous study (Van Och *et al*., 2000).

METHODS

Chemicals

DEA (diethylamine; 99.5%; free base solution; Sigma-Aldrich Chemie B.V., Zwijndrecht NL), TMTD (tetramethyl thiuram disulfide; 98%; Sigma-Aldrich) and ZDMC (zinc dimethyl dithiocarbamate; 90%; Fluka, Zwijndrecht NL) were tested in 4:1 acetone/olive oil (AOO). For both intradermal injection and topical treatment during the induction phase and for topical challenge in the guinea pig maximization test, the chemicals were dissolved in maize oil. Freund's complete adjuvant (FCA) was purchased from Sigma-Aldrich.

Table 1
Selection of the concentrations used in the main test, based on the results of the preliminary test

Compound	Induction*		Challenge*
	Intradermal	Topical	Topical
DEA	0.3% - 0.01%	10% - 0.3%	30%, 10%, 3% and vehicle
TMTD	0.3% - 0.01%	30% - 1%	30%, 10%, 3% and vehicle
ZDMC	0.3% - 0.01%	30% - 1%	30%, 10%, 3% and vehicle

* Dilution in maize oil

The Guinea Pig Maximization Test (GPMT)

Preliminary Test

The irritation response to intradermal injection of various concentrations of the test substance (3, 1, 0.3 and 0.1%) was examined in three guinea pigs. A sufficiently large area of the flanks was clipped free from hair with electric clippers. Amounts of 0.1 ml of the selected concentrations were applied by intradermal injection. Approximately 24 hours after injection, the animals were examined for signs of irritation. The concentration causing slight to moderate irritation but otherwise well tolerated by the animals, is taken for intradermal injection of the test substance in the intradermal induction phase of the main study (see Table 1).

The irritation response to topical treatment of various concentrations of the test substance (30, 10, 3 and 1%) was examined in three other guinea pigs. The flanks of each of the animals were clipped free from hair with electric clippers. Patches (Silverpatch, ½x½ cm, van der Bend B.V., Brielle, the Netherlands) were loaded with the test material and placed on the clipped skin of each animal, and covered with a piece of hypoallergenic paper bandage (Leukopor) that was secured by elastic adhesive tape (Tensoplast, T.J. Smith and Nephew Ltd., Hull, UK). The dressing was left in place for approximately 24 hours. Approximately 24 and 48 hours after removal of the dressing, the animals were examined for signs of skin irritation. The concentration causing slight to moderate skin irritation is chosen for topical induction and a non-irritant concentration for topical challenge (see Table 1).

Main Test

The experiments were conducted according to the maximization test method as described by Magnusson and Kligman (1969, and 1970), and according to OECD guideline no. 406 (1992) and EC guideline B.6 (1992). Approximately 3-wk-old, male SPF-outbred albino guinea pigs (Crl:(HA)BR) with a body weight range of 175-300 g, were purchased from Charles River (Wiga, Sulzfeld, Germany). They were housed in groups of no more than 10 in a mobile battery (IFFA CREDO, Someren, The Netherlands), containing four cages with internal dimensions of 967 x 512 x 250 mm in a temperature- and humidity-controlled animal room with a 12-hr light/dark cycle. Water and food (SDS Special Diets Services, Witham, UK) were provided *ad libitum*. The animals were kept in quarantaine for at least 1 week before the start of the study. During this period the health status was checked by clinical observation and serological examinations.

Briefly, the maximization test consists of two induction treatments, namely intradermally and topically, followed by a resting period of 14 days, which precedes the challenge treatments. Between induction treatments, a 7-day interval was employed. For the induction phase, dose response studies were performed with a fixed ratio (100 for TMTD and ZDMC and 33 for DEA) of concentrations between the intradermal and topical administration (Table 1).

Induction

Induction was performed in two different ways, firstly by intradermal injection and secondly, one week later, by topical application over the injection sites.

a. Intradermal injections

For this purpose an area of about 24 cm^2 of dorsal skin in the scapular region was clipped free from hair with electric clippers. Pairs of intradermal injections (0.1 ml each) were made simultaneously in the clipped area. The following preparations were injected:

test animals
- two injections with Freund's Complete Adjuvant (FCA) and saline (1:1),
- two injections with the selected test concentration,
- two injections with the selected test concentration and FCA/saline (1:1)

control animals
- two injections with FCA/saline (1:1),
- two injections with the vehicle,
- two injections with the vehicle and FCA/saline (1:1)

b. Topical application

Six days after the intradermal injections, the dorsal skin in the scapular region of all test and control animals was closely clipped again. On the following day, the induction by topical application was performed. The test animals were treated as follows: an approximate 2 x 4 cm patch of Whatman No. 3 MM filter paper was loaded with a fixed amount of the selected concentration of the test substance. The loaded patch was placed over the sites of the intradermal injections. The patch was covered hypoallergenic paper bandage (Leukopor) which was then secured by elastic adhesive bandage (Tensoplast), 7.5 cm in width, wound around the torso of the animal. The dressing was left in place for approximately 48 hours. The control animals were similarly treated with vehicle alone. Skin readings were made directly after removal of the patches.

We consider the intradermal and topical phase as the integral induction, and wanted to study dose response relations of induction and resulting effect. Hence, we kept the ratio of intradermal and topical induction constant for each compound.

Challenge
The topical challenge with the test substance was carried out 14 days after the start of topical induction as follows: an area of 5 x 5 cm on both flanks of each test and control animal was clipped free from hair. Patches (Silverpatch, ½x½ cm) were loaded with a fixed amount of the test concentration(s) selected and with the vehicle. Subsequently, the loaded patches were placed on the clipped area of the flanks of each test and control animal. The patches were covered with Leukopor bandage, and held in place by Tensoplast for approximately 24 hours. Every animal received all three challenge concentrations as well as vehicle alone (Table 1).

Evaluation
Skin reactions after challenge were evaluated at 24 and 48 hours after removal of the patches containing the test materials. The following ordinal grading scale was used: 0=no visible change, 1=discrete or patchy (slight) erythema, 2=moderate and confluent erythema, 3=intense (severe) erythema and swelling (Magnusson and Kligman, 1970). The positive results were evaluated according to the EC-standards (1993), which states that a substance is considered a sensitizer if 30% or more of the test animals show a positive reaction.

To study the potency of sensitizers in the GPMT, we determined dose-response relationships. The scores per animal ranged from 0 to 3. We used 5 animals per group; hence the scores per group could range from 0-15. We used a total score of 5 (TS_5), i.e. an average score of 1, the minimal receivable positive response for each animal, as a cut off value to establish the benchmark concentration. The highest score per animal read after either 24 or 48 hours was used in the statistical analysis. One animal in the lowest dose group of ZDMC died intercurrently during the experiment. In that particular dose group a total score of 4, instead of 5, was used as a cut off value to establish the benchmark concentration.

Local lymph node assay (LLNA)
The modified LLNA as previously conducted (van Och et. al., 2000) is described here. Groups of mice (n=3) were pretreated with 1% SDS (w/v) one hour before exposing the animals to 25 µl of test solution in vehicle or vehicle alone on both ears daily for three consecutive days. A positive response is not seen at the SDS concentration that we used (1%). However, application of 1% SDS and the test chemical generally resulted in an increased response compared to the test chemical alone (data not shown). Three days following the last topical application, the auricular lymph nodes were excised. The lymph nodes were weighed and pooled for each animal and suspended in 5 ml RPMI- 1640 (Gibco, Breda, NL) supplemented with 5% heat inactivated Fetal Calf Serum (PAA, Linz, Austria), 100 U/ml penicillin and 100 µg/ml streptomycin (standard medium). Single cell suspensions were prepared under aseptic conditions by pressing the lymph node through a sterile 70-µm nylon cell strainer (Falcon, Franklin Lakes, USA). The cells were washed twice in standard medium (10 minutes, 311g, 4°C) and resuspended in 1 ml standard medium with 10% FCS. The cells were counted using a Coulter Counter (Coulter Electronics, Mijdrecht, NL) and cultured at a concentration of 1.10^7 cells/ml. When necessary, cell suspensions of several animals were pooled to obtain the concentration required. The cell suspensions (200 µl) were seeded in triplicate into round-bottomed 96-well microtitre plates (Greiner, Alphen a/d Rijn, NL). The cells were cultured with 10 µl of [^{3}H]TdR (Amersham, Buckinghamshire, UK; 37 kBq/ml) for 24 hr at 37°C in a humidified atmosphere of 5% CO_2 in air. The [^{3}H]TdR incorporation was determined by liquid

scintillation counting in a β plate counter (1205 Betaplate™ Wallac, Turku, Finland). The [^{3}H]TdR incorporation is expressed per animal; i.e. the [^{3}H]TdR incorporation is multiplied by the cell number of the two lymph nodes and devided by the cell number in culture.

To study the potency of sensitizers, we have used a range of test concentrations to determine dose-response relationships on which the quantitative estimation of the allergic potency was based. The estimated concentration in % required for SI=3 (EC_3) was determined as the estimated dose inducing a stimulation index of three between treated versus control.

RESULTS

The mean scores for erythema and oedema as well as the summation over all animals of the highest score per animal, are shown for DEA, TMTD and ZDMC in tables 2-4, respectively. Because topical application of 30% DEA showed irritation in one of the test animals in the preliminary test (data not shown), a 10%-0.3% range was used in the induction phase for this compound, whereas a 30%-1% range was used for TMTD and ZDMC. The concentrations used during the intradermal phase was 100-fold higher for TMTD and ZDMC and 33-fold higher for DEA compared to the topical induction. As the intradermal induction phase is the dominant determinant of sensitization and topically sensitization only minimally contributes to the sensitization rate (Andersen *et al.*, 1984; Rohold *et al.*, 1991), we have plotted the intradermal induction concentration on the horizontal axis (Figure 1).

Table 2
Results of the GPMT with DEA (n=5). The positive number of animals and total skin response are shown. The highest score read after 24 or 48 hours was taken. The total score (TS) is the summation of each score for all animals. Every animal received all three challenge concentrations as well as vehicle alone.

	Challenge concentrations											
Induction	30%			10%			3%			vehicle		
Concentrations (%)[a]	24h	48h	TS	24h	48h	TS	24h	48h	TS	24h	48h	TS
0.3 + 10	2	3	5	1	1	2	0	1	1	0	0	0
0.1 + 3	3	3	4	1	1	3	0	1	1	0	0	0
0.03 + 1	2	0	3	1	0	1	1	0	1	0	0	0
0.01 + 0.3	4	1	4	0	0	0	1	0	1	0	0	0
0 + 0	0	0	0	0	0	0	0	0	0	0	0	0

[a] intracutaneous and topical, respectively, see Table 1

No skin reactions were seen in the control group after administration of 30% DEA in the challenge. Both TMTD and ZDMC showed positive responses in the control groups after a challenge application of 30%. Therefore, we used data derived after challenge administration of 10% for both chemicals where none of the control animals showed positive skin reactions. In addition, no skin responses were seen after application of vehicle alone in the challenge phase, in any of the animals tested (Table 2-4).

The maximal skin scores for each animal were summed per dose group, resulting in a total skin score. All three chemicals showed a decline of the number of positive animals after decreasing the challenge concentration as well as less severe responses per animal resulting in a decrease in the total skin score. The potency of the sensitizing capacity, based on the effective concentrations (i.e. those causing minimal detectable skin reactions in the GPMT), were compared to the potency as derived in a modified LLNA, performed in a previous study (Van Och *et al.*, 2000). These estimated concentrations after intrapolation at a total skin score of 5 (TS_5) are summarized in Table 5. The chemicals are ranked according to their TS_5 values.

Table 3
Results of the GPMT with TMTD (n=5). The positive number of animals and total skin response are shown. The highest score read after 24 or 48 hours was taken. The total score (TS) is the summation of each score for all animals. Every animal received all three challenge concentrations as well as vehicle alone.

Induction	Challenge concentrations											
	30%			10%			3%			vehicle		
Concentrations (%)[a]	24h	48h	TS	24h	48h	TS	24h	48h	TS	24h	48h	TS
0.3 + 30	5	5	12	4	5	7	3	2	4	0	0	0
0.1 + 10	5	5	11	4	4	8	4	3	6	0	0	0
0.03 + 3	5	4	8	3	1	4	1	0	1	0	0	0
0.01 + 1	2	2	2	1	0	1	0	1	1	0	0	0
0 + 0	4	2	4	0	0	0	0	0	0	0	0	0

[a] intracutaneous and topical, respectively, see Table 1

The data formerly obtained with the LLNA, being the EC_3 values of [^{3}H]TdR incorporation per animal (van Och et. al., 2000), are also shown in Table 5. The lowest TS_5 value derived from analysis of the GPMT dose response data was seen for ZDMC (0.01%), followed in this order by TMTD (0.05%) and DEA (0.30%). The ranking according to the estimated TS_5 values is somewhat different compared to the classification formerly obtained with the LLNA. The allergen DEA showed to be the least potent sensitizer in both tests. The allergen ZDMC showed a stronger response than TMTD in the GPMT performed here, but the sensitizing potential of ZDMC in the LLNA was weaker compared to the sensitizing response of TMTD.

Table 4
Results of the GPMT with ZDMC (n=5). The positive number of animals and total skin response are shown. The highest score read after 24 or 48 hours was taken. The total score (TS) is the summation of each score for all animals. Every animal received all three challenge concentrations as well as vehicle alone.

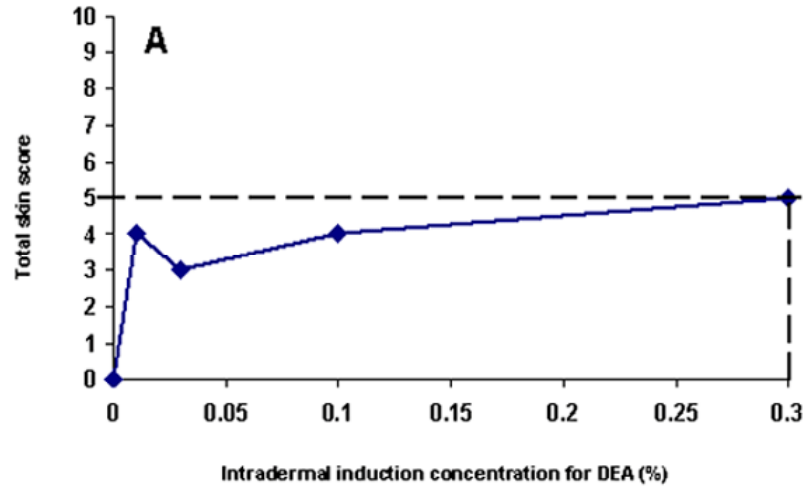
Induction	Challenge concentrations											
	30%			10%			3%			vehicle		
Concentrations (%)[a]	24h	48h	TS	24h	48h	TS	24h	48h	TS	24h	48h	TS
0.3 + 30	4	5	11	4	5	9	2	2	4	0	0	0
0.1 + 10	5	3	9	4	0	7	0	0	0	0	0	0
0.03 + 3	5	4	9	4	4	6	1	1	2	0	0	0
0.01 + 1[b]	3	3	6	2	1	4	1	1	2	0	0	0
0 + 0	4	3	6	0	0	0	0	0	0	0	0	0

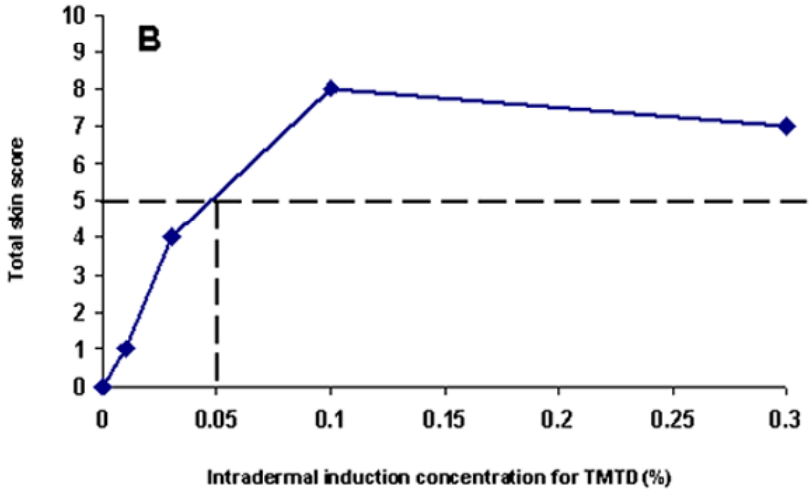
[a] intracutaneous and topical, respectively, see Table 1
[b] n=4, one animal died

DISCUSSION

In the GPMT, ZDMC showed the strongest sensitizing potency followed by TMTD and DEA. The LLNA showed that the sensitizing potential of TMTD was strongest, followed by ZDMC and DEA (van Och et. al., 2000). Hence, the ranking of the chemicals in the LLNA is not the same as the ranking according to their sensitizing potency in the GPMT as shown in Table 5.

Both GPMT and LLNA are used for risk assessment of sensitizing chemicals, but the question which of the tests is more relevant for the assessment of risk in man, remains to be established. The fact that ZDMC is a more potent sensitizer compared to DEA in man was confirmed in a recent article by Knudsen et. al. (2000). In the latter paper, the distribution of positive patch test reactions to rubber accelerators was evaluated in 25 patients. For ZDMC, 8


A
Total skin score
0
1
2
3
4
5
6
7
8
9
10
0
0.05
0.1
0.15
0.2
0.25
0.3
Intradermal induction concentration for DEA (%)


B
Total skin score
0
1
2
3
4
5
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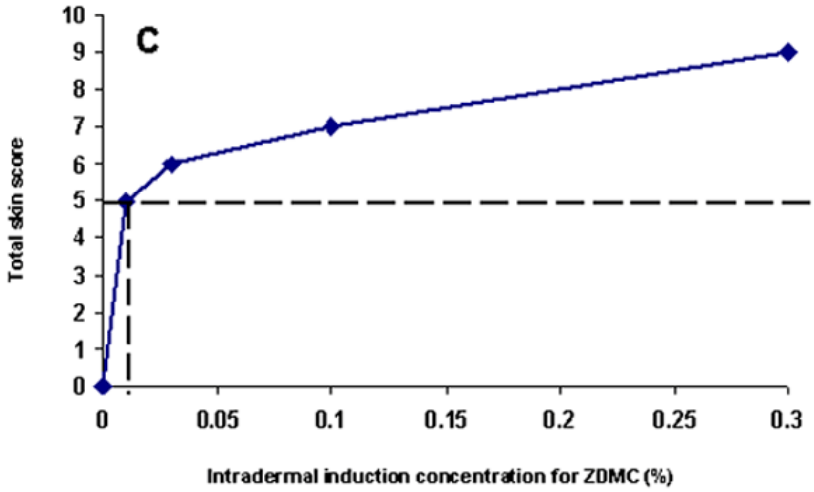
7
8
9
10
0
0.05
0.1
0.15
0.2
0.25
0.3
Intradermal induction concentration for TMTD (%)


C
Total skin score
0
1
2
3
4
5
6
7
8
9
10
0
0.05
0.1
0.15
0.2
0.25
0.3
Intradermal induction concentration for ZDMC (%)

out of 25 patients showed a positive patch test reaction while 1 out of 25 patients showed a positive patch test reaction for DEA. This finding is in accordance with our findings both in the GPMT and the LLNA.

In our view, including a dose-response analysis in the GPMT provides us a more accurate assessment of the potency of sensitizing chemicals. It enables a better estimation of the lowest concentration needed for sensitization. More reliable results may be obtained by increasing the number of concentrations investigated, by simultaneously reducing the number of animals per group. This allows testing of a wider range of doses and will give a better characterization of the shape of the dose response curve. Of course, it should be kept in mind that the actual risk for humans to develop allergy depends on many factors. Besides the concentration, the frequency and duration of exposure and the condition of the human skin are important factors.

A drawback of the GPMT, compared to the LLNA, is that skin scores are provided on an ordinal scale. Although dose-response modelling of ordinal data is by itself possible, the results of such an analysis are less easily interpreted. Further, ordinal data contain less information than quantitative (continuous) measures, such as the observed [^{3}H]TdR incorporation in the LLNA test. Indeed, dose-response modelling of the ordinal data from the GPMT was not as successful as the dose-response modelling of the LLNA results, and we decided to apply the simple method of plotting the sum of the scores against the induction dose. Clearly, this rough method results in an estimate of a benchmark dose that is less precise than that resulting from the analysis of the LLNA results. For that reason it may be hypothesized that the ranking of sensitizing chemicals is more reliable when based on a LLNA.

As cut off point we arbitrarily chose 5, which signifies the sum per group of the average minimal positive skin score of the animals. A skin score of 1 is the minimal positive score in the GPMT. It should be mentioned that the TS_5 concentrations derived in the GPMT are much lower compared to the EC_3 values in the LLNA. This may imply that the GPMT is more sensitive than the LLNA due to the disparities in procedure; i.e. different species, use of adjuvant, both intradermal and topical application of the chemical in the GPMT versus topical application in the LLNA and use of different vehicles. This latter issue may be especially important for detection of weak sensitizers.

All three chemicals showed a decline in the number of positive animals as well as decreased severity after decreasing the challenge concentration, resulting in a decreased total skin score. Obviously, the choice of the challenge concentration is crucial for the outcome of the test. Both TMTD and ZDMC, showed negative skin responses after topical application of 30% in the preliminary test. However, in the main test, topical application of this concentration showed positive animals, i.e. both chemicals scored four out of five read after 24 hours. These disparities are most likely due to interexperimental variation. Using a range of challenge concentrations allowed us to use the maximal non-irritant challenge concentration to evaluate these results.

Figure 1
Dose response curves for DEA (a), TMTD (b) and ZDMC (c) using the GPMT with a multiple dose design. The dose response curves are based on maximal skin responses taken after 24 or 48 hours per animal. A challenge concentration of 10% was taken for ZDMC and TMTD, while a challenge concentration of 30% was taken for DEA. A total score of 5 (TS_5), i.e. an average score of 1 for each animal was used, as a cut off value to establish the benchmark concentration.

Table 5
The guinea pig maximization test (GPMT) derived concentrations (TS_5) versus the local lymph node assay (LLNA) derived EC_3.

Chemical	GPMT	LLNA[#]
	TS_5 (%)	EC_3 (%)
ZDMC	0.01	2.670
TMTD	0.05	0.659
DEA	0.30	39.784

[#] Van Och et al. (2000)
EC_3: estimated concentration in % required for SI=3.
TS_5: estimated (intradermal) concentration in % required for a total skin score of 5.

In conclusion, the sensitizing potential of DEA, TMTD and ZDMC was evaluated using a dose-response analysis for the GPMT with a multiple-dose-design and was compared to the outcome of the LLNA test for the same chemicals. The GPMT and LLNA differed in the benchmark concentrations for the chemicals tested. While both tests ranked DEA as the least potent allergen, the GPMT ranked ZDMC more potent than TMTD, the reverse being found in the LLNA. The LLNA gives a more quantitative result on which regression analysis can be performed. Yet, the GPMT appears to reveal sensitization at lower concentrations, and may therefore be more sensitive. Further validation with human data is therefore necessary.

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CHAPTER 4

RANKING OF ALLERGENIC POTENCY OF LATEX CHEMICALS IN A MODIFIED LOCAL LYMPH NODE ASSAY

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A modified local lymph node assay (LLNA) with ex vivo tritium thymidine (^{3}H-TdR) labelling of the proliferating lymph node cells was used for determination of the allergenic potency of chemicals used in the production of rubber for latex medical gloves. Fifteen chemicals, known to induce contact hypersensitivity reactions in man, including various thiuram, carbamate, and benzothiazole compounds and one amine were tested. The EC_3 (effective concentration inducing a threefold increase in proliferation of lymph node cells (stimulation index, SI=3)) was calculated with non-linear regression analysis, including a bootstrap method for determination of the 5%-95% confidence interval of the EC_3 value. This procedure identified 14 out of the 15 chemicals tested as sensitiser, while for one chemical, ZDBC, no EC_3 could be calculated due to low responses and a lack of a dose response relationship in the data obtained. The ranking order of the chemicals with increasing EC_3 values (and thus decreasing allergenic potency) was found to be: ZDEC, TMTD, TETD, ZPC, ZDMC, MBTS, PTD, TMTM, MBT, MBI, PTT, ZMBT, TBTD, DEA, and ZDBC. Our results indicate that the chemicals of choice for use in the production of natural rubber latex products would be for the thiuram compounds TBTD, for the carbamates ZDBC and for the benzothiazoles ZMBT. However, one has to be aware that besides potency also the total amount of residual chemical present in the final product is important for allergy induction.

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INTRODUCTION

The use of natural rubber latex products poses risks for the development of allergy. Latex products can induce both immediate type (IgE mediated type I) hypersensitivity due to the latex proteins and/or cellular mediated delayed type (type IV) hypersensitivity due to the chemicals present as residues. Chemicals are added to rubber to improve it, to prolong its life and to give it special properties. Some of the chemicals are added directly at harvesting of the latex sap as preservatives, others are added as vulcanizers, accelerators, retarders, and/or antioxidants (Cronin, 1980). The added chemicals have been well documented as contact sensitisers in experimental studies in animals and humans, as well as in clinical studies (Cronin, 1980; Duarte *et al.*, 1998; Estlander *et al.*, 1986; Kaniwa *et al.*, 1994a; Kligmann, 1966; Magnussen and Kligmann, 1969; Maurer *et al.*, 1979; Taylor, 1995; Wrangsjö and Meding, 1994). Rubber polymer itself is not allergenic (Cronin, 1980). In most cases sensitisation by rubber chemicals is related to the use of natural rubber latex gloves (Conde-Salazar *et al.*, 1993) but sensitisation from rubber shoes also has been reported (Kaniwa *et al.*, 1994b). Causality has been demonstrated by identifying the particular chemical in the rubber material, and the presence of a positive patch test in humans to the rubber material concomitantly with a positive patch test to the pure chemical (Fregert *et al.*, 1969; Rycroft *et al.*, 1992).

Few studies have dealt with quantitative determinations of rubber chemicals in rubber products (Emmet *et al.,* 1994; Hansson *et al.*, 1997; Kaniwa *et al.*, 1994a; Knudsen *et al.*, 1993). An extraction procedure and quantitative analysis for the most common rubber chemicals in rubber gloves has been described recently (Knudsen *et al.*, 2000a). There was no clear correlation between chemical content in latex gloves and positive patch test reactivity in patients (Knudsen *et al.*, 2000b). However, a tendency was seen for gloves with a low combined amount of chemicals in the extracts to give few positive reactions in the patients while gloves with a high amount of chemicals gave many positive reactions (Knudsen *et al.*, 2000b). In addition, cross reactivity can occur between various chemicals i.e. between thiurams and carbamates (Knudsen *et al.*, 2000b; Knudsen and Menné, 1996), and mixtures of chemicals can be present in the gloves.

The chemicals most often reported as contact sensitisers belong to the thiuram, carbamate, and mercaptobenzothiazole groups, but also guanidines and several antioxidants have occasionally been reported as sensitisers (Estlander *et al.*, 1994). The risk for sensitisation and the resulting contact hypersensitivity reactions can theoretically be eliminated by a reduction of exposure and/or a complete substitution of sensitisers with non-sensitisers (Cardin *et al.*, 1986; Rycroft *et al.*, 1992). This is, however, not compatible with the use of natural rubber products. The use of new technologies such as irradiation instead of the traditional vulcanisation process may, however, decrease the demand for accelerators (Wan Manshol, 1998). Also the use of products manufactured from alternative materials will reduce induction of allergy to rubber chemicals. However, this does not completely prevent sensitisation by rubber chemicals as the same accelerators and antioxidants may be used as in latex production. Substitution of strong allergens with weaker allergens also is a possibility. Comparison and rating of sensitisers has been performed using experimental models in guinea pigs (Andersen *et al.*, 1995; Wang and Suskind, 1988) and mice (Basketter *et al.*, 1999a; Ikarashi *et al.*, 1994; Van Och *et al.*, 2000). However, experiments systematically investigating a wide range of chemicals used in natural rubber latex products have not been reported.

The local lymph node assay (LLNA) is now commonly used for the identification of the sensitising activity of chemicals and the assay has been validated to the guinea pig maximization test (Basketter and Scholes, 1992; Basketter *et al.*, 1993; Kimber *et al.*, 1995). Test chemicals are applied to the dorsum of the ear and lymphocyte activation is determined by

measuring cell proliferation in the draining auricular lymph nodes, which can be done either by *in vivo* or *ex vivo* tritium-thymidine labeling of the cells (Kimber and Weisenberger 1989, Kimber *et al.* 1995, Van Och *et al.* 2000). Analysis of 134 chemicals tested in the local lymph node assay (LLNA), guinea pig maximisation test (GPMT) and/or with clear clinical evidence for human skin sensitisation potential, revealed that an EC_3 (effective concentration causing a stimulation index of 3 compared to vehicle control) in the LLNA is an acceptable threshold value for hazard identification (Basketter *et al.*, 1999a). In the local lymph node assay quantitative data are obtained on the induction phase of the immune response. So, based on the EC_3 value of sensitising chemicals an accurate assessment of sensitising potency is possible (Basketter *et al.*, 1999a; Van Och *et al.*, 2000).

In the LLNA responses of chemicals can be compared and a ranking order of weak, moderate or strong sensitisers can be established (Basketter *et al.*, 1999a; Basketter *et al.*, 1999b; Van Och *et al.*, 2000). This offers the opportunity to use the LLNA in selecting chemicals with low or minimal allergenic potency. In this study a modified LLNA (sodium dodecyl sulfate pretreatment and ex vivo cell labeling) was used for determination of the sensitising potential of 15 chemicals used during natural rubber latex production, and known to induce contact hypersensitivity reactions in man.

MATERIALS AND METHODS

Animals

Young adult (6-8 weeks of age) female BALB/c mice were obtained from the Central Animal Laboratory of the Institute. BALB/c mice show similar responses in the LLNA compared to CBA/Ca mice (Woolhiser *et al*, 2000). The animals were bred specified pathogen free. During the experiments the animals were housed barrier-maintained under conventional conditions in light-, humidity-, and temperature controlled rooms. All animals were housed in macrolon cages. The mice were fed chow pellets (Hope Farms, Woerden, The Netherlands) and water ad libitum.

All other husbandry conditions were maintained according to all applicable provisions of the following national laws: Experiments on Animals Decree, and Experiments on Animals Act.

Chemicals

The chemicals investigated (Table 1) belong to different groups of compounds used during latex rubber glove production such as thiurams, carbamates, benzothiazoles and amines. TMTD, TETD, ZDEC, MBT, MBI, MBTS, and DEA were obtained from Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands. ZDMC was obtained from Fluka, Zwijndrecht, The Netherlands. TMTM was obtained from Acros Organics, Geel, Belgium. TBTD, PTD, ZDBC, and ZPC, Robinson Brothers Limited, West Bromwich, UK, and PTT, ICN pharmaceuticals, Costa Mesa, CA, USA, and ZMBT, Semperit, Vienna, Austria, were kindly provided by Dr. C. Hametmer, Östereichisches Institut für biomedizinische Werkstofftechnik, Vienna, Austria.

All chemicals were dissolved in 4:1 aceton/olive oil (AOO), and dose response studies were performed with the LLNA, as previously reported (Van Och *et al.,* 2000).

Experimental design

The sensitising potency of the chemicals was investigated in a modified local lymph node assay using ex vivo labeling of the proliferating lymph node cells (Kimber and Weisberger 1989, Vandebriel *et al.*, 2000; Van Och *et al.*. 2000). One hour before the chemicals were applied 25

Table 1
Chemicals investigated for their sensitising potency in a modified local lymph node assay (LLNA).

Chemical	Abbreviation	Purity	Formula
Thiurams			
Tetramethylthiurammonosulfide	TMTM	practical grade	$C_6H_{12}N_2S_3$
Tetramethylthiuramdisulfide	TMTD	± 98%	$C_6H_{12}N_2S_4$
Tetraethylthiuramdisulfide	TETD	± 98%	$C_{10}H_{20}N_2S_4$
Tetrabutylthiuramdisulfide	TBTD	technical grade	$C_{18}H_{36}N_2S_4$
Dipentamethylenethiuramdisulfide	PTD	technical grade	$C_{12}H_{20}N_2S_3$
Dipentamethylenethiuramtetrasulfide	PTT	technical grade	$C_{12}H_{20}N_2S_6$
Carbamates			
Zinc dimethyldithiocarbamate	ZDMC	± 90% purity	$C_6H_{12}N_2S_4Zn$
Zinc diethyldithiocarbamate	ZDEC	technical grade	$C_{10}H_{20}N_2S_4Zn$
Zinc dibutyldithiocarbamate	ZDBC	technical grade	$C_{18}H_{36}N_2S_4Zn$
Zinc pentamethylenedithiocarbamate	ZPC	technical grade	$C_{12}H_{20}N_2S_4Zn$
Benzothiazoles			
2-Mercaptobenzothiazole	MBT	± 98%	$C_7H_5NS_2$
Zinc mercaptobenzothiazole	ZMBT	technical grade	$C_{14}H_8N_2S_4Zn$
2,2-Dibenzothiazyldisulfide	MBTS	99%	$C_{14}H_8N_2S_4$
Mercaptobenzimidazole	MBI	98%	$C_7H_6N_2S$
Amines			
Diethylamine	DEA	free base solution	$C_4H_{11}N$

µl of 1% sodium dodecyl sulfate (SDS, Merck BV, Amsterdam, The Netherlands) was applied epicutaneously to the dorsum of both ears to enhance responses to weak sensitisers (De Jong *et al.* manuscript in preparation). Additionally 25 µl of test solution or vehicle control was applied to the dorsum of both ears (50 µl per animal) of female BALB/c mice daily for three consecutive days (days 0, 1, and 2). At day 5 following start of treatment animals were sacrificed and draining (auricular) lymph nodes (LN) were excised. Isolated left and right LN's from each mouse were weighed, and single cell suspensions prepared using a cell strainer (Falcon, Franklin Lakes, NJ, USA). Cells were washed twice and suspended in RPMI 1640 (Gibco, Grand Island, NY, USA) culture medium supplemented with 10% heat inactivated Foetal Calf Serum (PAA, Linz, Austria), 100 IU/ml penicillin, and 100 µg/ml streptomycin, refferred to as supplemented medium. Cells were counted in a Coulter Counter (Coulter Electronics, Mijdrecht, the Netherlands) and adjusted to a concentration of $1x10^7$ cells/ml. When necessary cell suspensions of several animals were pooled in order to obtain cell concentrations of $1x10^7$ cells/ml, notably so for vehicle AOO treated controls.

Lymphocyte stimulation test

LN cell suspensions, 2 x 10^6 cells in 200 µl, were cultured in RPMI 1640 supplemented medium in triplicate in round-bottomed 96-wells microtitre plates (Greiner, Alphen aan de Rijn, The Netherlands). An aliquot of 10 µl of ^{3}H-methylthymidine, 37 kBq/well, (^{3}H-TdR, Amersham International, Buckinghamshire, UK; 3.7 MBq/ml, 100 µCi/ml), specific activity 185 GBq/mmol (5 Ci/mmol in 217.8 µg/ml cold thymidine in PBS) was added to the wells of the cell culture directly after initiation of culture. Cultures were maintained for 24 hours at 37 ^oC in a humidified atmosphere of 5% CO_2 in air. The cellular DNA was harvested on glass fiber filters using an automatic cell harvester (Harvester 96® Tomtec, Orange, CT, USA), scintillation liquid was added and incorporation of ^{3}H-TdR into the DNA was measured by liquid scintillation in a β plate counter (1205 Betaplate™ Wallac, Turku, Finland). Proliferation

per animal was determined by calculating the ^{3}H-TdR incorporation for the total cell number harvested (left and right lymph nodes combined).

Statistical analysis

The EC_3 (effective concentration inducing a threefold increase in ^{3}H-thymidine incorporation in the harvested lymph node cells of treated animals compared to vehicle treated animals) was estimated by the benchmark approach, by fitting a nonlinear regression model to the data of all individual animals. The choice of the model for deriving the EC_3 follows from a procedure of applying likelihood ratio tests on the members of the following nested family of models:

model 1: $y = a$
model 2: $y = a \exp(bx)$
model 3: $y = a \exp(bx^d)$
model 4: $y = a(c-(c - 1)\exp(bx))$
model 5: $y = a(c - (c - 1)\exp(bx^d))$,

where y is the response, and x denotes the applied concentration. The parameter a represents the level of the response at concentration zero, and b can be considered as the parameter reflecting the efficacy of the chemical. At high doses models 4 and 5 level off to the value ac, so the parameter c can be interpreted as the maximum relative change, compared to the background. Models 3 and 5 have the flexibility to mimic threshold-like responses. All these models are nested to each other, except models 3 and 4, which both have three parameters. Therefore, these two models cannot be (formally) compared to each other by a likelihood ratio test.

For each data set (compound) one of these models was selected by choosing a more complicated model when the increase in number of parameters resulted in a significantly better fit to the dose-response data by choosing a more complicated model when the increase in number of parameters resulted in a significantly better fit to the dose-response data. The selected model was used to estimate the EC_3 (point estimate). Additionally, an estimate of the uncertainty (90%-confidence interval) associated with the estimated EC_3 was determined using a (parametric) bootstrap method (Slob and Pieters, 1998), as follows. Once a model is selected for describing the dose-response data, this fitted model is used as a basis for generating 200 artificial data sets (according to the experimental design) by Monte Carlo sampling. For each generated data set the EC_3 is re-estimated. Taking all these EC_3s together results in a distribution representing the uncertainty associated with the EC3 estimate. The 5th and 95th percentiles of this empirical distribution were determined, serving as a 90%-confidence interval for the EC_3. Full statistical details are given in Slob (2001). Other applications can be found in Slob 1999, Piersma *et al*. 2000, Van Och *et al*., 2000, and Woutersen *et al*. 2001.

RESULTS

Dose response evaluations of two compounds are presented in Table 2. ZDEC induced a strong response for all parameters determined, while ZMBT induced a weak response. For ZDEC the lymph node weight, number of cells isolated per animal, and the ^{3}H-thymidine incorporation per cell culture ($2x10^6$ cells) and per animal (left and right lymph node combined) increased with higher dosages. For ZMBT only moderate changes were observed. Especially for the lower dosages of chemicals and the AOO control, lymph node cell populations had to be pooled in order to obtain a sufficient number of cells for the in vitro ^{3}H-thymidine labelling.

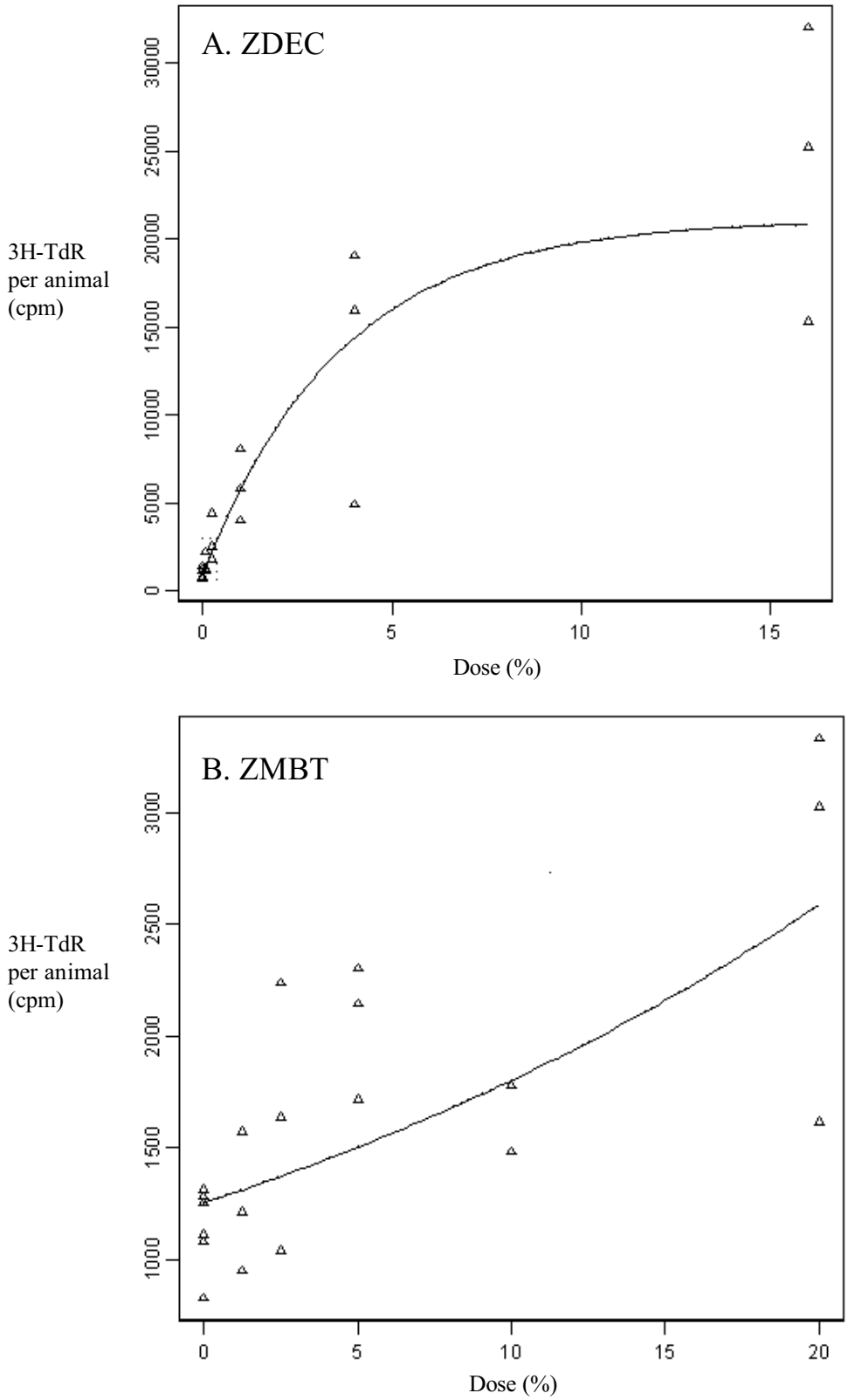

Figure 1
Observations (cpm) as a function of concentration for ZDEC (A) and ZMBT (B) together with fitted regression function.

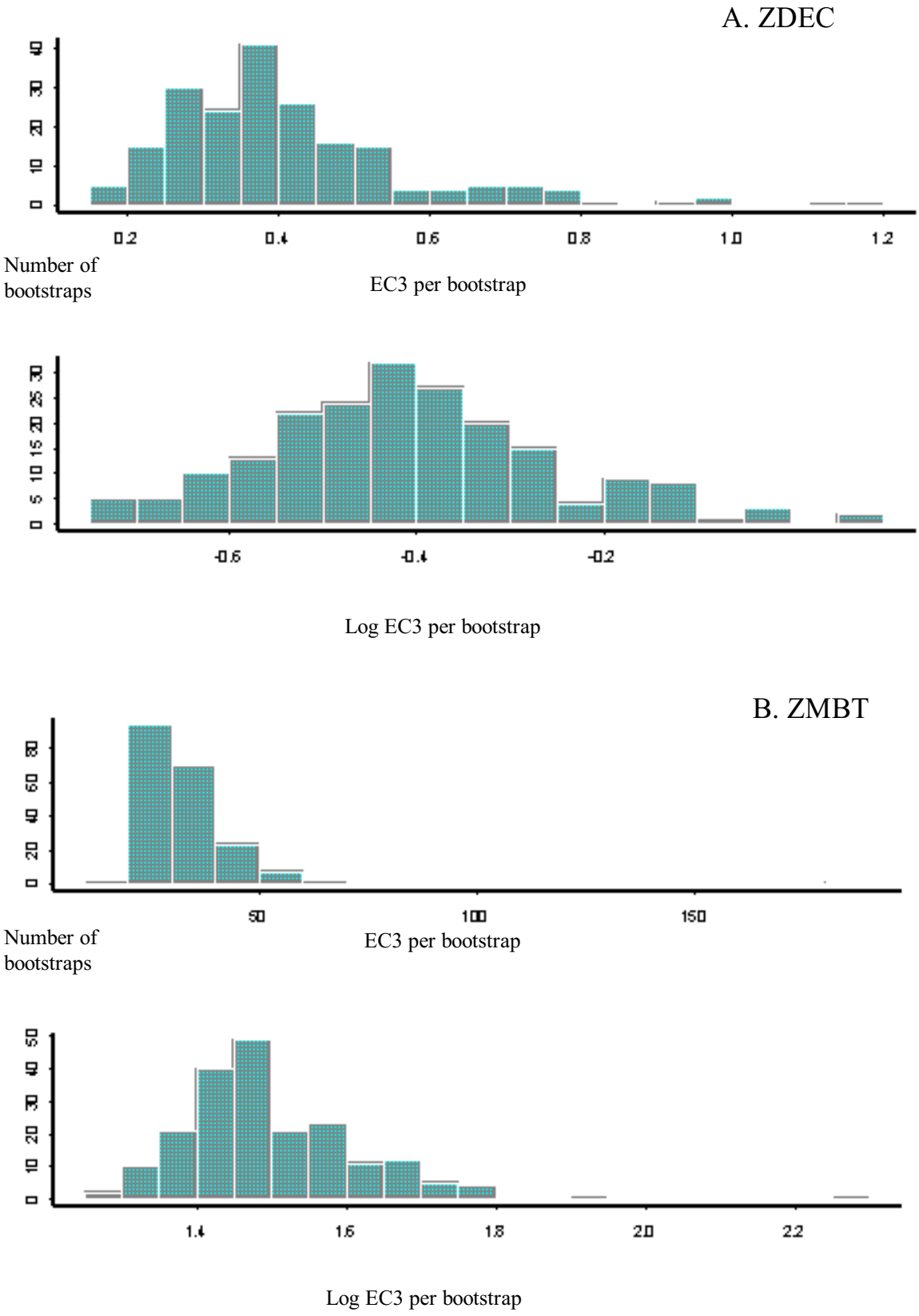

Figure 2
A. Distribution of EC_3 value of ZDEC (n=200). B. Distribution of EC_3 value of ZMBT (n=200).

Table 2
Dose response studies with the strong sensitiser ZDEC and the weak sensitiser ZMBT.

Chemical Concentration (%)	LN Weight (mg)	LN Cell Number x10e6	Proliferation cpm/2*10e6 LN cells	LN Proliferation cpm/animal
ZDEC				
0	3.1 ± 0.2 (12)[a]	5.4 ± 0.4 (6)	353 ± 68 (2)	960 ± 115 (6)
0.0625	2.7 ± 0.3 (6)	4.4 ± 1.0	711 (1)	1,565 ± 339
0.25	3.4 ± 0.4 (6)	6.6 ± 1.1	819 ± 344 (2)	2,936 ± 771
1	3.5 ± 0.3 (6)	8.9 ± 1.0	1,328 ± 194	5,976 ± 1163
4	5.7 ± 0.4 (6)	13.2 ± 2.2	1,923 ± 477	13,312 ± 4294
16	7.2 ± 0.8 (6)	16.3 ± 1.5	2,921 ± 376	24,197 ± 4834
ZMBT				
0	3.2 ± 0.2 (12)	3.6 ± 0.2 (6)	638 ± 3 (2)	1,144 ± 74 (6)
1.25	3.0 ± 0.2 (6)	3.8 ± 0.6	655 (1)	1,244 ± 180
2.5	3.8 ± 0.2 (6)	4.1 ± 0.9	799 (1)	1,638 ± 346
5.0	3.9 ± 0.2 (6)	3.8 ± 0.3	1073 (1)	2,056 ± 176
10.0	3.8 ± 0.2 (6)	3.7 ± 0.2	846 (1)	1,580 ± 99
20.0	4.4 ± 0.2 (6)	5.3 ± 1.0	1010 (1)	2,659 ± 529

[a] Arithmetic means and standard errors of the mean (SEM), (n) n=3 unless stated otherwise.

All individual animal data (expressed as proliferation per animal) were used to estimate the best fitting non linear regression model as determined with the maximum likelihood. For ZDEC and ZMBT the best fitting curve is presented in Figures 1A and 1B, respectively. The EC_3 concentration (concentration of the chemical inducing a SI=3) was estimated using a benchmark approach (see M&M), by fitting a dose-response model to the observed ^{3}H-thymidine incorporation as a function of the concentration of the chemical (Figure 1A and B). The EC_3 value for ZDEC (EC_3= 0.4%) is shown in Figure 1A, while for ZMBT the EC_3 value (EC_3= 30.3%) was calculated by extrapolation of the experimental results shown in Figure 1B.

The EC_3 value is an estimated value from a best fitting curve. To account for the noise in the data, a parametric bootstrap method was used for further evaluation of the EC_3 value. The results of 200 bootstrap runs indicating possible EC_3 values based on the experimental data obtained for ZDEC and ZMBT, are presented in Figures 2A and 2B. For ZDEC the EC_3 value ranges from 0.1% to 0.8%, while for ZMBT the EC_3 values ranges from 20% to 50%. With the 200 parametric bootstrap runs the 5% and 95% confidence limits of the EC_3 can be estimated. Table 3 shows the calculated EC_3 values and their 90% confidence intervals for 15 chemicals evaluated in the modified LLNA. For 14 out of the 15 chemicals tested an EC3 was determined, identifying these chemicals as sensitisers. For ZDBC the curve fitting and bootstrap method did not result in an EC_3 value due to low responses and the lack of a dose response relationship of the data obtained in the LLNA**.** Based on the EC_3 values the chemicals are ranked from high to low reactivity in the LLNA (Table 3).

DISCUSSION

We tested 15 different chemicals used in latex medical glove production. The chemicals were chosen because of their known sensitising capacity as they were found to induce contact hypersensitivity (DTH) reactions in humans (Knudsen *et al.*, 2000b). Fourteen of the 15 chemicals were positively identified, while the assay failed to detect ZDBC, a carbamate compound, as sensitiser. Using the LLNA we quantified the sensitising potency and found.

Table 3
Ranking of chemicals used for latex production.

Chemical	EC3[a]	L05 - -L95[b]	Ranking LLNA
ZDEC	0.4	0.2 - 0.6	1
TMTD	0.7	0.6 - 0.8	2
TETD	1.4	0.9 - 1.9	3
ZPC	1.8	0.7 - 6.0	4
ZDMC	2.7	1.6 - 8.3	5
MBTS	2.9	1.4 - 6.7	6
PTD	5.2	2.8 - 10.7	7
TMTM	5.4	1.9 - 11.5	8
MBT	9.9	8.0 - 12.2	9
MBI	14.7	11.8 - 19.8	10
PTT	20.5	11.6 - 66.0	11
ZMBT	30.3	22.2 - 49.0	12
TBTD	34.8	15.6 - 114	13
DEA	39.8	34.1 - 47.7	14
ZDBC	NR[c]		15

[a] Effective concentration inducing a stimulation index of 3 in LLNA
[b] Estimated 90%-confidence interval based on 200 bootstrap runs (see M&M)
[c] NR = EC3 not reached according to fitted model.

ZDEC the strongest and DEA the weakest sensitiser. The associated 90% confidence interval gives an indication of the reliability of the estimated EC_3 concentration. Use of the EC_3 values makes it possible to rank various chemicals used within a single production process, with regard to their sensitising potency (Basketter *et al.*, 1999a; Van Och *et al.*, 2000). This offers the possibility for manufacturers to choose chemicals with low sensitising properties. In the production process of natural rubber latex several different types of chemicals are used each with their own function in the production process (Cronin, 1980). Our results may provide information to make a choice between these chemicals. Especially chemicals failing to induce a SI=3 or needing rather high concentrations to induce a stimulation index of 3 seem warranted for use in latex production facilities. Our results indicate that in particular the chemicals of choice are for the thiuram compounds TBTD, for the carbamates ZDBC, and for the benzothiazoles ZMBT. Besides sensitising activity also the total amount present and the bio-availability determines the sensitising potency of a medical glove manufactured from natural rubber latex. The total amount of chemicals in glove extracts varies but is in the same order of magnitude (approximately 2-15 µmol/g glove), although tenfold differences between different manufacturers may occur (Knudsen *et al.*, 2000a).

There was no group of chemicals as a whole showing for all compounds either a low or high allergenicity as indicated by the EC_3 value. Our results suggest that for the thiuram compounds an increase in EC_3 value, and thus a decrease in sensitising potency, occurs with an increase in the lenght of the side chains. For TMTD (methyl side chains) the EC_3 was 0.7%, for TETD (ethyl side chains) the EC_3 was 1.4%, and for TBTD (butyl side chains) the EC_3 was 34.8%. For the corrsponding carbamates a similarity could be observed for ZDBC (butyl side chains) being non sensitising (EC_3 value could not be determined). In contrast ZDMC (methyl side chains, EC_3= 2.7%) was less potent than ZDEC (ethyl side chains, EC_3= 0.4%). The benzothiazoles showed EC3 values from low (MBTS EC_3= 2.9%) to high (ZMBT EC_3= 30.3%). For TMTD, ZDMC, MBT and DEA a decreasing order in sensitising potential has already been reported (Van Och *et al.*, 2000). Ikarashi *et al.*, (1993) reported a decreasing

order in sensitising activity for IPPD (N-isopropyl-N'-phenyl-*p*-phenylenediamine), TMTD, MBT and ZDEC. In contrast to their results we found ZDEC to be more potent than MBT. Furtheremore, for none of the doses investigated a SI=3 was reached for TMTD and ZDEC by Ikarashi *et al.*, (1993). The comparison of sensitising potency was based on the highest proliferation index obtained. In our view this is less accurate than comparison on the basis of the EC_3 value. Also the difference in vehicle used for ZDEC may have contributed to the low reactivity in the study of Ikarashi *et al.*, (1993). We used acetone olive oil, which is commonly used in the LLNA, whereas Ikarashi *et al.*, (1993) used chloroform. Although for strong sensitisers the influence of the vehicle is probably limited, it is known that the vehicle has an effect on the performance of the LLNA (Edwards *et al.*, 1994; Montelius *et al.*, 1996; Warbrick *et al.*, 1999).

Besides the qualification of a compound as sensitiser the use of non-linear regression analysis for calculation of the EC_3 offers the advantage of a quantitative estimation of the confidence limits of the estimated EC_3. Non linear regression analysis as applied in this study, uses all available individual data, and not just the mean of the treated animals. Thus, a 90%-confidence interval could be calculated. For the stronger sensitisers in general the 90%-percentile confidence interval is smaller than for the weak sensitisers (also on the log-scale, i.e. in a relative sense). The 5th percentile level may be interesting from the point of risk management, as this percentile indicates that below this level there is only a 5% probability that positive reactions do occur. So, the 5th percentaile of the EC_3 is very similar to the BMDL (lower confidence limit of the benchmark dose) and may be used as the lowest acceptable dose (or concentration for exposure) for risk management purposes. Comparing our results with clinical data (Knudsen *et al.*, 2000b), for the weakest identified chemicals a low frequency of positive reactions was found while for the strongest identified chemicals a high frequency of positive reactions was observed.

In conclusion, a ranking is presented for rubber chemicals known to induce contact hypersensitivity reactions in man. When using these chemicals for the production of natural rubber latex products, the preference would be to use chemicals with absent or low sensitising activity. Our results indicate that the chemicals of choice are for the thiuram compounds TBTD, for the carbamates ZDBC and for the benzothiazoles ZMBT.

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CHAPTER 5

EFFECT OF EXPOSURE DURATION ON IDENTIFICATION OF ALLERGENS IN A MODIFIED LOCAL LYMPH NODE ASSAY

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The local lymph node assay (LLNA) is an assay in mice to identify potential allergens. Compounds that do not induce a stimulation index (SI) ≥ 3 are not considered sensitizers. Of the chemicals that do, the SI of 3 is used as a benchmark, and indicates the sensitizing potency of a chemical. Compared to the exposure duration of the LLNA (3 days), real life exposure often lasts for months or years. We therefore investigated whether prolonged exposure to sensitizers at concentrations that do not induce an SI ≥ 3 in the LLNA, were able to surpass this threshold. Mice were treated for 2 months at 7-day intervals with a range of concentrations of the known allergens ethyl-p-aminobenzoate (benzocaine, BENZ), 2,4-dinitrochlorobenzene (DNCB), and tetramethyl thiuram disulfide (TMTD). Both proliferative activity and cytokine production were established at day 60. Neither BENZ nor TMTD showed a significant increase in the proliferation rate compared to vehicle controls. Only DNCB at concentrations originally above the EC_3 a significant increase in proliferation was seen after prolonged exposure. No significant effect on IFN-γ and IL-4 production was observed for all three compounds compared. These findings indicate that for classification of sensitizers the shorter exposure period in the LLNA is sufficient, and longer periods of exposure have no bearing on this classification.

Toxicology, in press

INTRODUCTION

Recently, the murine local lymph node assay (LLNA, Kimber et al., 1986; Kimber and Weisenberger, 1989) was submitted to the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) for consideration as a stand-alone test method alternative to accepted guinea pig test methods. It was concluded that the LLNA is an acceptable alternative to methods such as the guinea pig maximization test to assess whether a chemical has the potential to induce allergic contact dermatitis (ACD) in humans. The criterion for a positive LLNA response, and for classification of a chemical as a contact sensitizer, is a threefold or greater increase in lymph node (LN) cell proliferative activity compared to vehicle treated controls. The selection of a stimulation index (SI) of 3 for the definition of skin sensitization potential in the LLNA was empirical, being based on experience and experimental observations in humans (Basketter et al., 1999a).

Besides the identification, the relative potency of a potential contact allergen is of importance. Dose-response relationships in cellular proliferation results in a calculated EC_3 value (concentration inducing SI = 3) for the various chemicals (Basketter et al., 1999b). We have previously determined the relative potency in a quantitative manner using a modified LLNA (Van Och et al., 2000; De Jong et al., 2002a). For an SI = 3, we calculated the corresponding estimated concentration (EC_3) using non-linear regression analysis, resulting in ranking of the various chemicals tested. These dose-response data also show that for each chemical a concentration exists which does not induce a significant response. Besides experimental evidence in the LLNA, also studies in humans suggest the presence of thresholds for the induction of skin sensitization (Steltenkamp et al., 1980; Danneman et al., 1983; Ford et al., 1988).

Depending on the situation, humans are exposed to allergens almost every day. In most cases it concerns very low exposure levels at which people will not develop a specific allergic response. Occasionally, someone will become allergic. Previous studies have indicated that the frequency of antigen exposure is an additional risk factor in determining the outcome of the immune response (Mueller et al., 1989; Kitagaki et al., 1995). An important question that arises is at which time point this change will occur and what determines a change from non-reaction to reaction. For instance, humans are continuously exposed to nickel originating from a variety of sources. Skin contact with nickel-containing or nickel-plated tools and other items adds to an occupational nickel hazard and leads eventually to contact dermatitis or nickel allergy (Grandjean, 1984). The potential threshold for inducing nickel sensitivity in humans was investigated by Allenby and Basketter (1993; 1994). Individuals were exposed by arm immersion twice daily for 23 days to a surfactant solution; 60% of the people tested showed positive reactions to 10 ppm aqueous nickel sulfate, 50% to 5 ppm, 15% to 1 ppm, and 10% to 0.5 ppm. In contrast, a relatively high single exposure to 100 ppm showed a reaction in none of the 51 individuals tested (Menneé and Calvin, 1993). So, the actual risk for humans to develop allergy depends not only on concentration, but also on frequency of exposure. Other factors are the condition of the human skin, the anatomical site of exposure, and genetic background.

The cytokines induced in the LLNA can be used to discriminate contact allergens from respiratory sensitizers (Dearman et al., 1994; Vandebriel et al., 2000). Both studies showed that compounds inducing contact hypersensitivity (CHS) produce relatively high levels of IFN-γ and low levels of IL-4. So, in addition to the proliferative responses we also investigated the influence of prolonged exposure on cytokine production. Animals were treated for 2 months at 7-day intervals with the chemicals ethyl-p-aminobenzoate (benzocaine, BENZ), 2,4-dinitrochlorobenzene (DNCB), and tetramethyl thiuram disulfide (TMTD). These compounds have been well documented as contact sensitizers in experimental studies in animals, as well as

in clinical observations (Kligman and Epstein, 1959; Magnusson and Kligman, 1969; Cronin, 1980; Basketter and Scholes, 1992; Kaniwa et al., 1993). The concentrations used were chosen below and above their respective EC_3 values that were formerly obtained with a modified LLNA (Van Och et al., 2000). Both proliferative activity and cytokine production were established on day 60.

METHODS

Animals

Young adult (6-8 weeks old) male or female BALB/c strain mice were used for the experiments. They were obtained from our own breeding colony. The animals were bred specific pathogen free and kept under conventional conditions. The mice were fed Hope Farms chow pellets (Woerden, NL) and water *ad libitum*. The breeding colony of the animals was pre-screened/monitored for endogenous pathogenic viruses and bacteria and was found negative.

The experimental setup of the studies was examined and agreed upon by the Ethical Committee on Experimental Animals.

Chemicals

Benzocaine (BENZ, ethyl-p-aminobenzoate; 99% purity; Sigma-Aldrich Chemie B.V., Zwijndrecht, NL), DNCB (2,4-dinitrochlorobenzene; 98%; Sigma-Aldrich), and TMTD (tetramethylthiuramdisulfide; 98%; Sigma-Aldrich) were dissolved in 4:1 acetone/olive oil (AOO). SDS (sodium dodecyl sulphate; >99%; Merck B.V., Amsterdam, NL) was dissolved in 4:1 acetone/olive oil (AOO).

The concentrations for the different chemicals were chosen below and above the EC_3 as previously determined in a modified LLNA (Van Och et al., 2000, see Table 1A).

Table 1A
Experimental design for determination of effect of prolonged exposure with low concentrations on local lymph node activation. Table A shows the different concentrations used in a modified LLNA and the prolonged exposure study, the study design is shown in Table B

Chemical	EC_3 (%) in LLNA[a]	% concentration (w/v)	
		LLNA (short-term)[a]	Long-term exposure
BENZ	22.0	7.5, 15, 22.5, 30	7.5, 15, 22.5, 30
DNCB	0.044	0.1, 0.25, 0.5, 1	0.01, 0.025, 0.05, 0.1
TMTD	0.66	0.0312, 0.0625, 0.125, 0.25, 0.5, 1	0.0312, 0.0625, 0.125, 0.25

[a] EC_3; estimated concentration in % required inducing an SI=3 as previously determined in a modified LLNA (Van Och et al., 2000)

Sensitization and elicitation procedure

Groups of mice (n=5 for the chemicals, and n=10 for the AOO vehicle control group) were exposed to 25 µl of test solution in vehicle or vehicle alone on the dorsum of both ears (50 µl/animal) daily for three consecutive days (day 0, 1, and 2), and subsequently weekly on day 7, 14, 21, 28, 35, 42, 49, and 56. On day 0, 1, and 2 the animals were pretreated with 1% SDS

(w/v) on the dorsum of both ears one hour before exposing the animals on both ears. SDS treatment was performed to compare the present data with previous observations (Van Och et al., 2000; De Jong et al., 2002b). An overview of the study design is shown in Table 1B.

Table 1B

Day	1% SDS pretreatment	Allergen painting	Excision LN
0, 1, 2	+	+	-
7, 14, 21, 28, 35, 42, 49, 56	-	+	-
60	-	-	+

Cell proliferation

Four days following the last topical application (day 60), the auricular lymph nodes (LN) were excised. The LN were weighed and pooled for each animal and suspended in 5 ml RPMI-1640 (Gibco, Life Technologies, Breda, NL) supplemented with 5% heat inactivated Fetal Calf Serum (PAA, Linz, Austria), 100 U/ml penicillin and 100 µg/ml streptomycin (standard medium). Single cell suspensions were prepared under aseptic conditions by pressing the LN through a sterile 70 µm nylon cell strainer (Falcon, Franklin Lakes, NJ, USA). The cells were washed twice in standard medium (10 minutes, 311g, 4°C) and resuspended in 1 ml standard medium with 10% FCS. The cells were counted using a Coulter Counter (Z2, Coulter Electronics, Mijdrecht, NL) and cultured at a concentration of 1.10^7 cells/ml. When necessary, cell suspensions of several animals were pooled to obtain the concentration required. The cell suspensions (200 µl) were seeded in triplicate into round-bottomed 96-well microtitre plates (Greiner, Alphen a/d Rijn, NL). The cells were cultured with 10 µl of [^{3}H]TdR (Amersham International, Buckinghamshire, UK; 3.7 MBq/ml) for 24 hr at 37°C in a humidified atmosphere of 5% CO_2 in air. The [^{3}H]TdR incorporation was determined by liquid scintillation counting in a β plate counter (1205 Betaplate™ Wallac, Turku, Finland). The [^{3}H]TdR incorporation is expressed per animal, i.e. the [^{3}H]TdR incorporation is multiplied by the cell number of the two LN and devided by the cell number in culture.

Cytokine production

LN cell suspensions were made as described above. The suspensions were cultured at 10^6 cells per ml culture medium with Con A (5 µg/ml) and 5 x 10^{-5}M 2-mercaptoethanol for 24 hr. Con A stimulation proved necessary for the detection of IFN-γ and IL-4 (Vandebriel et al., 2000). After culture, aliquots of supernatants were frozen on dry ice and stored at -80 °C until use. IFN-γ and IL-4 concentrations were determined by ELISA. Briefly, 96-well plates (Nunc-Immuno Plate, Roskilde, Denmark) were coated with 1 µg/ml anti-mouse IFN-γ (R4-6A2, rat IgG_1; Pharmingen, San Diego, CA, USA) in coating buffer (0.04 M carbonate buffer, pH 9.6). After overnight incubation at 4°C the plates were incubated in blocking buffer (1% bovine serum albumin (BSA; Sigma, Axel, NL) plus 0.05% Tween-20 (Merck, Amsterdam, NL), in PBS) for 2 hrs at 37°C and washed (0.05% Tween-20). Recombinant mouse IFN-γ (Biosource, Camarillo, CA, USA) was used as a standard. Standard as well as serial dilutions of culture supernatants were added to the plate. Plates were incubated at 37°C for 2 hrs and washed. Biotinylated anti-mouse IFN-γ (0.5 µg/ml; XMG1.2, rat IgG_1; Pharmingen) was added and incubated for 1 hr at room temperature (RT). The plates were washed, and poly horseradish peroxidase labeled streptavidin (10,000-fold dilution, Strepta-E+, Central Laboratory of the Blood Transfusion Service, Amsterdam, NL) was added and incubated for 1 hr at RT. Plates were washed again and TMB solution (0.1 mg/ml TMB

(Sigma) plus 0.006% H_2O_2, in 0.1 M NaAc, pH 5.5) was added. The plates were read at 450 nm, using an automated reader (Titertek Multiskan, Flow Laboratories, Lugano, Switzerland). For IL-4, a similar protocol was used, with 0.5 μg/ml anti-mouse IL-4 (11B11, rat IgG_1; Pharmingen) for coating, recombinant mouse IL-4 (Pharmingen) as a standard, and 0.5 μg/ml biotinylated anti-mouse IL-4 (BVD6-24G2, rat IgG_1; Pharmingen) for detection. Antibodies, standards, samples, and streptavidin were diluted in 0.5% BSA plus 0.05% Tween-20, in PBS (Van Halteren et al., 1997).

Data are expressed as mean (ng) ± SEM. Similar to cell proliferation, results are expressed per two LN (per animal), i.e. the response (ng/ml) is multiplied by the local LN cell number, and divided by the cell culture concentration (10^6/ml).

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the two-tailed Student's *t* test using the ANOVA calculated pooled standard deviation. Significance levels were (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$ for proliferation data and cytokine production.

For DNCB, the proliferation data were analyzed using non-linear regression, as was previously described (Van Och et al., 2000; De Jong et al., 2002a).

Table 2
IFN-γ and IL-4 production after long-term exposure to DNCB. BALB/c mice were pretreated with 1% SDS by topical application on both ears and one hour later treated by topical application on both ears with various concentrations DNCB for three consecutive days (day 0, 1, and 2) and then the chemicals were repeatedly applied to the originally sensitized ears from day 7 to day 56 at 7-day intervals. Four days following the final topical application (day 60), the auricular LN were excised. LN cell suspensions were made, and IFN-γ and IL-4 concentrations were determined by ELISA. Results are expressed per two LN (per animal). Results are calculated by multiplying the cytokine concentration by the LN cell number, and are expressed as amount of cytokine (pg) ± SEM (n=5)

	IFN-γ	IL-4	IFN-γ/IL-4
AOO	N.D.	18 ± 5	-
0.01%	N.D.	21 ± 4	-
0.025%	85 ± 31	25 ± 3	3.4
0.05%	6779 ± 5525	791 ± 476	8.6
0.075%	9760 ± 7968	578 ± 588	16.8

N.D., not detected

RESULTS

LN cell proliferation

Figure 1 shows the dose-response relationships of the LN cell proliferation for the different chemicals after prolonged exposure. The concentrations used were chosen below and/or above their respective EC_3 values that were formerly obtained with a modified LLNA (Table 1). Proliferation was measured 4 days following the last application (at day 60) of BENZ, DNCB, and TMTD. For TMTD all concentrations used were < EC_3 and showed no differences in proliferation compared to AOO vehicle treated animals. Also no induction of the proliferative activity of the LN cells was found for BENZ, even for 22.5% and 30% BENZ, being concentrations > EC_3. DNCB induced significantly higher proliferative responses relative to

vehicle treatment for the two highest concentrations used ($p < 0.01$ for 0.05%, and $p < 0.05$ for 0.075%), both concentrations being > EC_3. DNCB concentrations < EC_3 did not induce proliferation. The concentration inducing an SI = 3 over vehicle control was calulated by non-linear regression analysis together with the corresponding 90%-confidence interval. The EC_3 value in the present study (0.092%) was two-fold higher than the concentration obtained in a modified LLNA (0.044%). No overlap between the corresponding 90%-confidence intervals (0.079%-0.122% and 0.025%-0.078%, respectively) was observed.

Table 3
IFN-γ and IL-4 production for DNCB obtained with a modified LLNA. BALB/c mice were pretreated with 1% SDS by topical application on both ears and one hour later treated by topical application on both ears with various concentrations DNCB for three consecutive days. Five days following the first topical application, the auricular LN were excised. LN cell suspensions were made, and IFN-γ and IL-4 concentrations were determined by ELISA. Results are expressed per two LN (per animal). Results are calculated by multiplying the cytokine concentration by the LN cell number, and are expressed as amount of cytokine (pg) ± SEM (n=4). (*), (**), and (***), significantly different from AOO ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively)

	IFN-γ	IL-4	IFN-γ/IL-4
AOO	157 ± 50	N.D.	-
0.1%	359 ± 321	22 ± 30	16.3
0.25%	27940 ± 11443**	916 ± 281	30.5
0.5%	76791 ± 8743***	2394 ± 207***	32.1
1%	163893 ± 20829***	2950 ± 660***	55.6

N.D., not detected

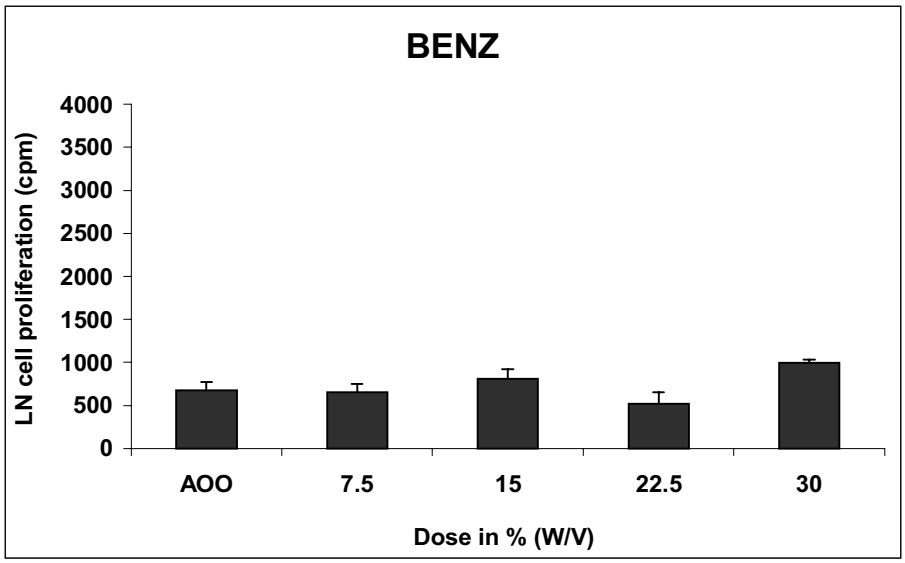
Cytokine production

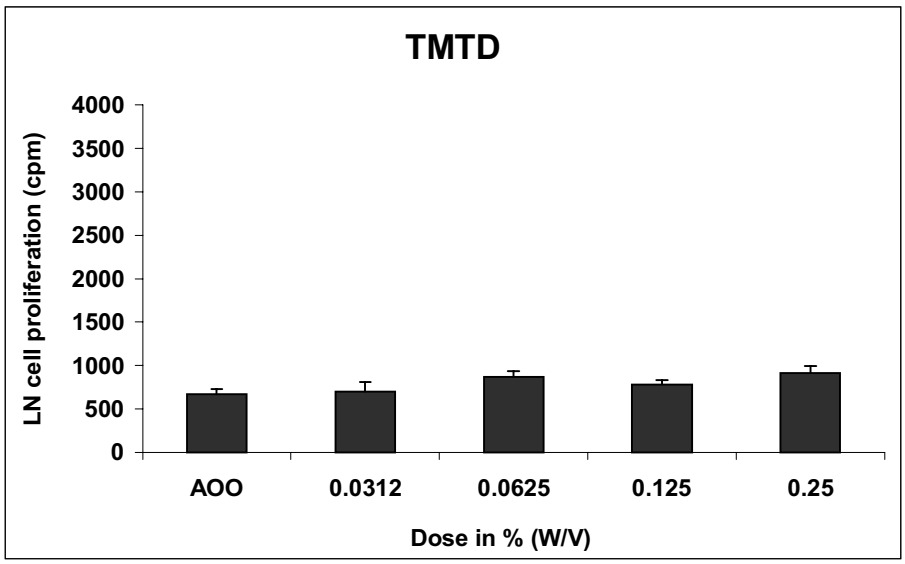
The production of IFN-γ and IL-4 was measured after prolonged exposure (2 months) to BENZ, DNCB, and TMTD. After prolonged exposure, only DNCB induced detectable amounts of IFN-γ and IL-4 (Table 2). However, the cytokine production was reduced compared to (short-term) 3-day exposure. These findings were compared to cytokine production derived from a modified LLNA after (short-term) 3-day exposure (Table 3). Remarkably, the IFN-γ/IL-4 ratio increased with increasing dose.

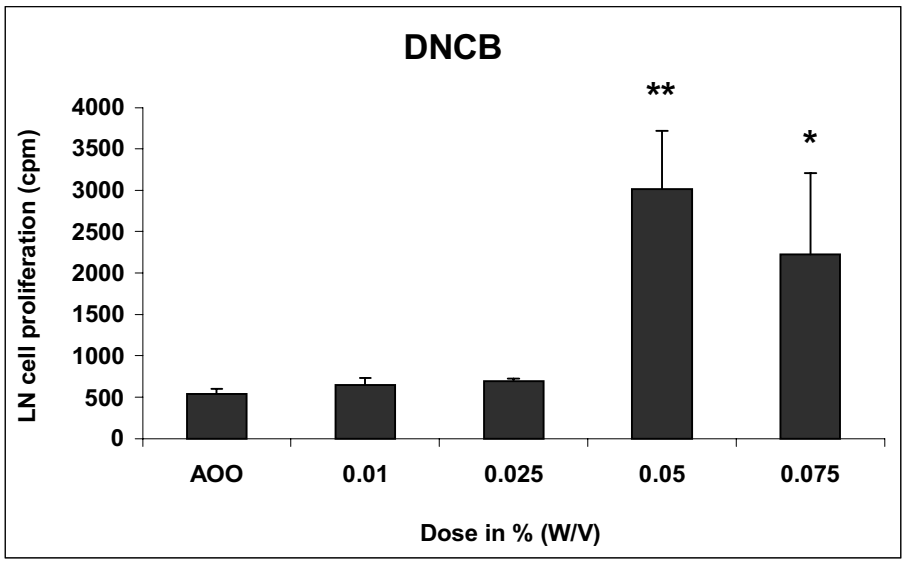
DISCUSSION

In the present study, we investigated if prolonged (2 months) exposure to low concentrations of allergens that do not induce an SI ≥ 3 in the LLNA, were able to surpass this threshold. To this end, the concentrations used were chosen around their respective EC_3 values that were formerly obtained with a modified LLNA (Van Och et al., 2000). The EC_3 values derived for

Figure 1
LN cell proliferation after long-term exposure. BALB/c mice were treated by topical application on both ears with various concentrations BENZ, DNCB, and TMTD for three consecutive days and then the chemicals were repeatedly applied to the originally sensitized ears from day 7 to day 56 at 7-day intervals. Four days after the last application, the local LN were excised, pooled (per animal for allergen treatment and per 2 animals for vehicle treatment), and cell suspensions were prepared. Cells were cultured for 24 hrs in the presence of ^{3}H-thymidine, and ^{3}H-incorporation was measured. Results are calculated by multiplying the ^{3}H-incorporation by the LN cell number, and expressed as cpm ± SEM (n=5, except AOO control group n=10). (*), (**), significantly different from AOO ($P < 0.05$, $P < 0.01$, respectively).


BENZ
LN cell proliferation (cpm)
4000
3500
3000
2500
2000
1500
1000
500
0
AOO
7.5
15
22.5
30
Dose in % (W/V)


TMTD
LN cell proliferation (cpm)
4000
3500
3000
2500
2000
1500
1000
500
0
AOO
0.0312
0.0625
0.125
0.25
Dose in % (W/V)


DNCB
**
*
LN cell proliferation (cpm)
4000
3500
3000
2500
2000
1500
1000
500
0
AOO
0.01
0.025
0.05
0.075
Dose in % (W/V)

BENZ, DNCB, and TMTD in a modified LLNA were 22.03%, 0.044%, and 0.66%, respectively. The data shown in the present paper are the results of three independent experiments.

First, TMTD, with all concentrations chosen below the EC_3 value (see Table 1A), was examined. Prolonged exposure did not result in induction of proliferation with an SI > 3. For the second compound studied, BENZ, we chose two concentrations above the EC_3 value and the other concentrations below this value. Again, none of the concentrations tested showed a proliferation rate with an SI > 3. Even 22.5 % and 30% BENZ, concentrations above the EC_3 value formerly obtained with a modified LLNA, showed no induction of proliferation. Because BENZ showed variable results in the past in both the GPMT and the LLNA (Basketter et al., 1995), we performed a third experiment, using the more potent allergen DNCB. After prolonged exposure, DNCB showed significantly higher proliferative responses relative to vehicle treatment for the highest two concentrations used, both concentrations being above the EC_3 value for DNCB. Moreover, the EC_3 value obtained in the present study (0.092%), was higher than the concentration found in a modified LLNA (0.044%; Van Och et al., 2000). No overlap was found between the corresponding confidence intervals of the EC_3 values, since the 90%-confidence interval as obtained in a modified LLNA for EC_3= 0.044% was [0.025%, 0.078%], and the 90%-confidence interval found in the present study for EC_3= 0.092% was [0.079%, 0.122%]. So, none of the concentrations used that are known to induce LN cell proliferation below an SI = 3, induced a significant increase in LN cell proliferation after prolonged exposure.

Besides proliferation, IFN-γ and IL-4 production were determined. Again, no significant induction of cytokine production for the different chemicals was seen after prolonged exposure to concentrations of allergens that did not induce an SI ≥ 3 in a modified LLNA. Even concentrations that did surpass this threshold, such as 0.05% DNCB and 0.075% DNCB, showed no significant increase in cytokine production compared to vehicle treated control animals. However, although not significantly increased for these two highest concentrations, cytokine production was clearly induced (Table 2).

Regarding the qualitative effect on the response (T-helper 1/T-helper 2 ratio, measured as IFN-γ/IL-4 ratio), the highest DNCB concentration in the long-term exposure study (0.075%, Table 2) showed an equivalent ratio compared to the lowest DNCB concentration in a modified LLNA (0.1%, Table 3). So, no evidence was found for a shift in the T-helper 1/T-helper 2 ratio depending on exposure time. However, longer exposure time did show a quantitative effect. Both 0.05% DNCB and 0.075% DNCB induced considerable amounts of IFN-γ and IL-4 compared to the production seen in the draining LN cells after exposure to 0.1% DNCB in a modified LLNA. This was not caused by the increase in LN cell number in time, because the same effect was found for cytokine production on a per cell basis (data not shown).

Remarkably, the ratio between IFN-γ and IL-4 showed a clear dose-dependency for DNCB, i.e. in favour of T-helper 1 at higher concentrations of DNCB. This confirms the proposal that it is neccessary to study a range of concentrations instead of only a single concentration, for an allergen to be identified as a contact sensitizer or as a respiratory allergen (Van Och et al., submitted).

In conclusion, for risk evaluation it is neccessary to estimate the lowest concentration needed for sensitization. We investigated the influence of exposure duration on the EC_3 value, since this parameter is the criterion for classification of a chemical as a contact sensitizer in the LLNA. For the three chemicals tested, none of the concentrations used that are known to induce LN cell proliferation below an SI = 3, induced a significant increase in LN cell proliferation after prolonged exposure. So, prolonged exposure to concentrations of allergens

below an SI of three in the LLNA, did not result in a response in the draining LN and thus were not able to surpass this threshold.

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CHAPTER 6

CYTOKINE PRODUCTION INDUCED BY LOW MOLECULAR WEIGHT CHEMICALS AS A FUNCTION OF THE STIMULATION INDEX IN A MODIFIED LOCAL LYMPH NODE ASSAY: AN APPROACH TO DISCRIMINATE CONTACT SENSITIZERS FROM RESPIRATORY SENSITIZERS

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Rob J. Vandebriel

In general, contact sensitizers have been shown to selectively induce Th1 immune responses, such as IFN-γ production, whereas Th2 responses, such as IL-4 production, were seen after exposure to respiratory allergens. However, these features may be dependent on the dose of the particular allergen. Therefore, the aim of the present study was to investigate the distinction between contact sensitizers and respiratory allergens, by establishing dose-dependent cytokine profiles. The contact allergens 2,4- dinitrochlorobenzene (DNCB), hexyl cinnamic aldehyde (HCA) and oxazolone (OXA, 4-ethoxymethylene 2- phenyloxazol-5-one), as well as the respiratory allergens fluorescein 5-isothiocyanate (FITC), phthalic anhydride (PA), toluene diisocyanate (TDI), and trimellitic anhydride (TMA) were tested. For a range of concentrations, both proliferative responses and cytokine production were established. Estimated concentrations were derived at several stimulation indices (SI's). From the estimated concentrations, IFN-γ, IL-4, and IL-10 production as a function of stimulation indices were plotted. All four respiratory allergens showed significantly higher IL-4 and IL-10 production patterns compared to the contact allergens. Positive identification of DNCB, HCA, and OXA as contact allergens on the basis of IFN-γ production, was observed only at very high stimulation indices (SI≥35) for DNCB and OXA and already at low SI's (SI≤7) for HCA. We propose that by direct linkage of proliferation and cytokine production in a dose-response manner, distinguishing contact allergens from respiratory allergens may be improved compared to present approaches.

Toxicology and Applied Pharmacology, in press

INTRODUCTION

The murine local lymph node assay (LLNA) is a method for the predictive identification of chemicals that have the potential to cause sensitization (Kimber *et al*., 1995; Loveless *et al*., 1996). In this test, the lymph node (LN) proliferative responses of treated animals are compared to those of vehicle-treated animals. Chemicals that elicit a stimulation index (SI) of 3 or more in the LLNA are considered as being sensitizers. In addition, these EC_3 (estimated concentration in % required for SI=3) values can be used for the comparison of sensitizing potential derived from local LN responses (Kimber *et al*., 1995; Kimber and Basketter, 1997; Loveless *et al*., 1996). Besides linear evaluation the dose response can be evaluated by nonlinear regression analysis. This latter approach was previously taken by us using a modified LLNA (Van Och *et al*., 2000), where the concentration inducing an SI of three is estimated based on a curve fitting method. In addition, confidence limits provide information on the reliability of the data.

However, the LLNA as such does not discriminate between contact and respiratory allergens. Another, experimentally used test in mice is also based on dermal exposure, but in contrast to the LLNA comprises both a sensitizing and effector phase (Dearman *et al*., 1994, 1995, 1996ab). This test showed that contact allergens preferentially induce a T-helper 1 (TH1) response, whereas respiratory allergens preferentially induce a T-helper 2 (TH2) response. These responses can be discriminated on the basis of cytokine production, such as IFN-γ, that is produced by TH1 cells, and IL-4 and IL-10, that are produced by TH2 cells.

In a previous study, using a modified LLNA (Vandebriel *et al*., 2000), we tried to establish whether the LLNA proved sufficient not only to identify allergens, but also to mark them as either a contact or a respiratory allergen. To compare the different chemicals, concentrations were chosen that induced similar levels of LN cell proliferation. Our findings showed that respiratory allergens can be identified on the basis of IL-4 production, since application of the respiratory allergens phthalic anhydride (PA), toluene diisocyanate (TDI), and trimellitic anhydride (TMA) resulted in a 15- to 30-fold higher IL-4 production compared to the contact allergen 2,4- dinitrochlorobenzene (DNCB). Application of TDI, TMA, and PA induced similar amounts of IFN-γ compared to DNCB and thus IFN-γ seemed not suitable as a positive indicator for the identification of contact allergens.

The approaches referred to above are comparisons based on single concentrations. However, linking cytokine production to proliferation of the same cells over the entire range of concentrations used may provide a more complete insight in the capacity of sensitizers to induce cytokines. Therefore, dose-response studies in a modified LLNA were performed for both proliferative responses and cytokine production after exposure to the contact allergens DNCB, HCA, and OXA, as well as the respiratory allergens FITC, PA, TDI, and TMA. From that, using non-linear regression analysis for both parameters individually, models describing proliferative responses vs. cytokine production were plotted. By evaluating the cytokine profile based on the proliferative response, it can be established whether an unknown allergen is a contact or respiratory allergen. In addition, since this approach directly links cytokine production to proliferation of the same cells, this approach is excluding the need for parallel, dissimilar experiments. The approach presented in this study is a way to discriminate between contact allergens and respiratory allergens independently from the choice of the concentrations used. The use of a single short assay for both proliferation (for identification of sensitizers) and cytokine production (for discrimination between sensitizers) is in line with one of the three R's (reduction, refinement, and replacement) of animal welfare policies.

METHODS

Chemicals

DNCB (2,4-dinitrochlorobenzene; 98%; Sigma-Aldrich, Zwijndrecht, the Netherlands), FITC isomer I (Fluorescein 5-isothiocyanate; 90%; Sigma-Aldrich), oxazolone (OXA, 4-ethoxymethylene 2- phenyloxazol-5-one; 90%; Sigma-Aldrich), PA (phthalic anhydride; 99%; Sigma-Aldrich), TDI (toluene 2,4-diisocyanate; 99.8%; Sigma-Aldrich), and TMA (trimellitic anhydride; 1, 2, 4- benzenetricarboxylic anhydride; 97%; Sigma-Aldrich), were dissolved in 4:1 acetone/olive oil (AOO). HCA (hexyl cinnamic aldehyde; 85%; Sigma-Aldrich) was dissolved in 4:3:3 dimethylacetamide/acetone/ethanol (DAE). AOO and DAE were used as vehicle control.

Animals

Male BALB/c mice were used at the age of 6-8 weeks. They were obtained from our own breeding colony. The diet consisted of ground standard laboratory chow (RMH-B, Hope Farms, Woerden, the Netherlands). Food and water were given *ad libitum*. The breeding colony of the animals was pre-screened/monitored for endogenous pathogenic viruses and bacteria and was found negative.

The experimental setup of the studies was examined and agreed upon by the Ethical Committee on Experimental Animals.

Modified local lymph node assay (LLNA)

Groups of mice (n=4, n=8 for the AOO control group, except HCA, n=6 for test groups and control group) were exposed to 25 µl of test solution in vehicle or vehicle alone on both ears daily for three consecutive days. Five days following the first topical application, the auricular lymph nodes were excised. The lymph nodes (LN) were weighed and pooled for each animal and suspended in 5 ml RPMI- 1640 (Gibco, Breda, the Netherlands) supplemented with 5% heat inactivated Fetal Calf Serum (PAA, Linz, Austria), 100 U/ml penicillin and 100 µg/ml streptomycin (standard medium). Single cell suspensions were prepared under aseptic conditions by pressing the LN through a sterile 70 µm nylon cell strainer (Falcon, Franklin Lakes, NJ). The cells were washed twice in standard medium (10 minutes, 311g, 4°C) and resuspended in 1 ml standard medium with 10% FCS. The cells were counted using a Coulter Counter (Coulter Electronics, Mijdrecht, the Netherlands) and cultured at a concentration of 1.10^7 cells/ml. LN cells from allergen treated animals were seeded for individual animals, while LN cells from AOO treated animals were seeded from pooled animals (2 animals). The reason to pool LN cells from different animals was that in case of AOO treated animals the number of LN cells obtained per animal was insufficient to perform the subsequent assays. The cell suspensions (200 µl) were seeded in triplicate into round-bottomed 96-well microtitre plates (Greiner, Alphen a/d Rijn, the Netherlands). The cells were cultured with 10 µl of [^{3}H]TdR (Amersham, Little Chalfont, United Kingdom; 37 kBq/ml) for 24 hr at 37°C in a humidified atmosphere of 5% CO_2 in air. The [^{3}H]TdR incorporation was determined by liquid scintillation counting in a β plate counter (1205 Betaplate^{TM} Wallac, Turku, Finland). The [^{3}H]TdR incorporation is expressed per animal, i.e. the [^{3}H]TdR incorporation is multiplied by the cell number of the two lymph nodes and divided by the cell number in culture ($2x10^6$).

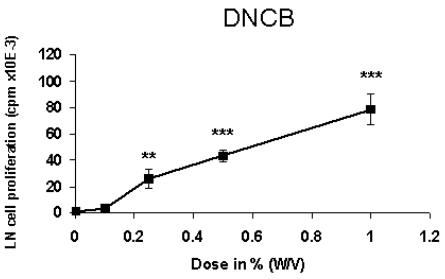
We have used a range of test concentrations to determine dose-response relationships. The estimated concentration in % required for a certain SI=*x* (EC_x) was determined as the estimated dose inducing a stimulation index of *x* between treated versus control animals.

Table 1
Concentrations used in the modified local lymph node assay (LLNA)

Chemical	% Concentration (w/v)
DNCB	0.1, 0.25, 0.5, 1
FITC	0.025, 0.1, 0.25, 1
HCA	3, 10, 30
OXA	0.0025, 0.01, 0.025, 0.1
PA	0.25, 1, 2.5, 10
TDI	0.1, 0.25, 1, 2.5
TMA	1, 2.5, 5, 10

ELISA.
LN cell suspensions were made as described above. The suspensions were cultured at 10^6 cells per ml culture medium with Con A (5 μg/ml) and 5 x 10^{-5}M 2-mercaptoethanol for 24 hrs. After culture, aliquots of supernatants were frozen on dry ice and stored at -80 °C until use. IL-4, IL-10 and IFN-γ concentrations were determined by ELISA. Briefly, 96-well plates (Nunc-Immuno Plate, Roskilde, Denmark) were coated with 1 μg/ml anti-mouse IFN-γ (R4-6A2, rat IgG_1; Pharmingen, San Diego, CA) in coating buffer (0.04 M carbonate buffer, pH 9.6). After overnight incubation at 4°C the plates were incubated in blocking buffer (1% bovine serum albumin (BSA; Sigma, Axel, the Netherlands) plus 0.05% Tween-20 (Merck, Amsterdam, the Netherlands), in PBS) for 2 hrs at 37°C and washed (0.05% Tween-20). Recombinant mouse IFN-γ (Biosource, Camarillo, CA) was used as a standard. Standard as well as serial dilutions of culture supernatants were added to the plate. Plates were incubated at 37°C for 2 hrs and washed. Biotinylated anti-mouse IFN-γ (0.5 μg/ml; XMG1.2, rat IgG_1; Pharmingen) was added and incubated for 1 hr at room temperature (RT). The plates were washed, and poly horseradish peroxidase labeled streptavidin (10,000-fold dilution, Strepta-E+, Central Laboratory of the Blood transfusion service, Amsterdam, the Netherlands) was added and incubated for 1 hr at RT. Plates were washed again and TMB solution (0.1 mg/ml TMB (Sigma) plus 0.006% H_2O_2, in 0.1 M NaAc, pH 5.5) was added. The plates were read at 450 nm, using an automated reader (Titertek Multiskan, Flow Laboratories, Lugano, Switzerland). For IL-4, a similar protocol was used, with 0.5 μg/ml anti-mouse IL-4 (11B11, rat IgG_1; Pharmingen) for coating, recombinant mouse IL-4 (Pharmingen) as a standard, and 0.5 μg/ml biotinylated anti-mouse IL-4 (BVD6-24G2, rat IgG_1; Pharmingen) for detection. The IL-10 content

Figure 1
Effect of topical application on lymphocyte proliferation. BALB/c mice were treated by topical application on both ears with various concentrations DNCB, FITC, HCA, OXA, PA, TDI, and TMA for three consecutive days. Five days after the first application, the local LN were excised, pooled (per animal for allergen treatment and per 2 animals for vehicle treatment), and cell suspensions were prepared. Cells were cultured for 24 hrs in the presence of ^{3}H-thymidine, and ^{3}H-incorporation was measured. Results are calculated by multiplying the ^{3}H-incorporation by the LN cell number, and expressed as cpm ± SEM (n=4). (*), (**), (***), significantly different from AOO ($P < 0.05$, $P < 0.01$, $P < 0.001$, respectively).


DNCB
LN cell proliferation (cpm x10E-3)
Dose in % (W/V)
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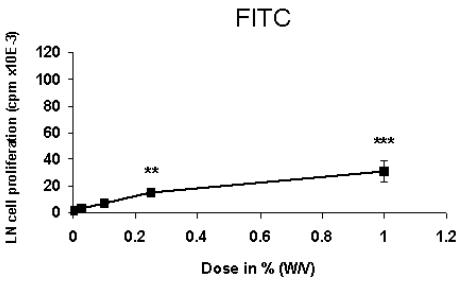
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Dose in % (W/V)
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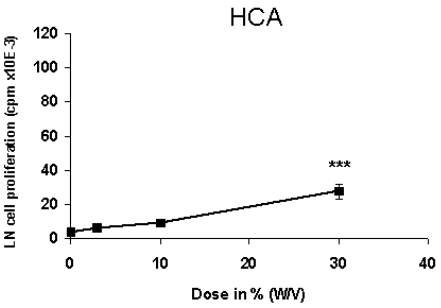
HCA
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Dose in % (W/V)
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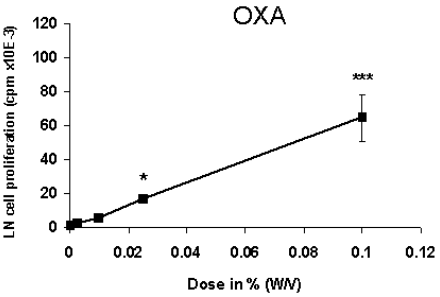
OXA
LN cell proliferation (cpm x10E-3)
Dose in % (W/V)
*
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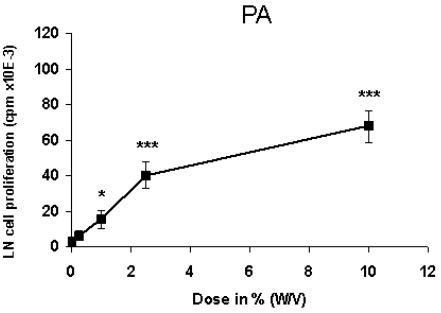
PA
LN cell proliferation (cpm x10E-3)
Dose in % (W/V)
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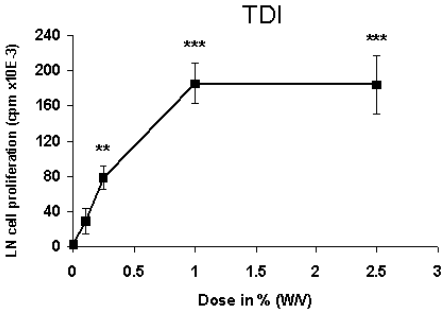
TDI
LN cell proliferation (cpm x10E-3)
Dose in % (W/V)
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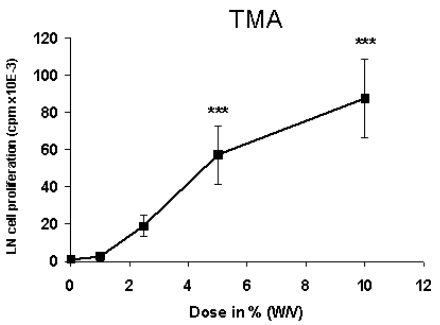
TMA
LN cell proliferation (cpm x10E-3)
Dose in % (W/V)
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***

of culture supernatants was measured using 4 μg/ml anti-mouse IL-10 (JES052A5, rat IgG_1; R&D Systems, Oxon, UK) for coating and 400 ng/ml biotinylated goat-anti-mouse IL-10 (IgG; R&D Systems) for detection. Recombinant mouse IL-10 (R&D Systems) was used as a standard. Antibodies, standards, samples, and streptavidin were diluted in 0.5% BSA plus 0.05% Tween-20, in PBS (Van Halteren *et al.,* 1997).

Data are expressed as mean (ng) ± SEM. Similar to cell proliferation, results are expressed per two LN (per animal), i.e. the response (ng/ml) is multiplied by the local LN cell number, and divided by the cell culture concentration (10^6/ml).

Statistical analysis

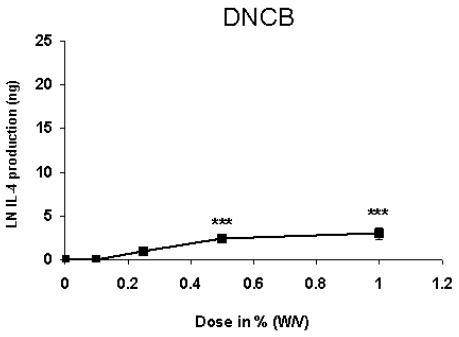
Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the two-tailed Student's *t* test using the ANOVA calculated pooled standard deviation. Significance levels were (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$.

The dose-response data were analyzed by nonlinear regression analysis, using the following family of models:

model 1: $y = a$
model 2: $y = a \exp(b x)$
model 3: $y = a \exp(b x^d)$
model 4: $y = a (c - (c - 1) \exp(b x)$
model 5: $y = a (c - (c - 1) \exp(b x^d))$
model 6: $y = d + a (x^c/(b + x^c))$

where y represents the response and x the applied concentration. In these models the parameter *a* represents the background of the particular assay. In all models the parameter *a* is constrained to being larger than zero, since it denotes the value of the response at dose zero. The parameter *b* reflects the 'slope' or the 'strength' of the response with increasing dose. Parameter *c* determines whether the function increases or decreases, by being larger or smaller than unity, respectively. To render model 3 describing a decreasing response *d* has to be changed into *-d*. The parameter *d* is constrained to values larger than (or equal to) one, to prevent the slope of the function at dose zero from being infinite, this being biologically unreal. The selection of the model to be used for a particular data set follows from a procedure of successively fitting the above models, and applying likelihood ratio tests to establish if an increase in the number of parameters leads to a significantly better fit to the data. A model with more parameters is considered better only if this leads to a significantly better fit (Slob, 1999). The selected model is used to derive the estimated concentration (EC) associated with a stimulation index or cytokine production. Full statistical details are given in Slob (2002).

Figure 2
Effect of topical application on IL-4 production. BALB/c mice were treated by topical application on both ears with various concentrations DNCB, FITC, HCA, OXA, PA, TDI, and TMA for three consecutive days. Five days following the first topical application, the local LN were excised, pooled per animal, and cell suspensions were prepared. Cells were cultured for 24 hrs in the presence of Con A and supernatants were analyzed for cytokine content using ELISA. Results are calculated by multiplying the IL-4 concentration by the LN cell number, and expressed as amount of IL-4 (ng) ± SEM (n=4). (**), and (***), significantly different from AOO ($P < 0.01$, and $P < 0.001$, respectively).


DNCB
LN IL-4 production (ng)
Dose in % (W/V)
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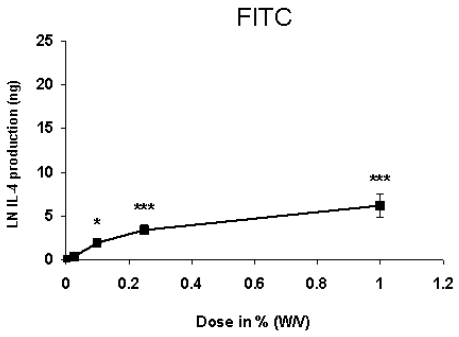
FITC
LN IL-4 production (ng)
Dose in % (W/V)
*
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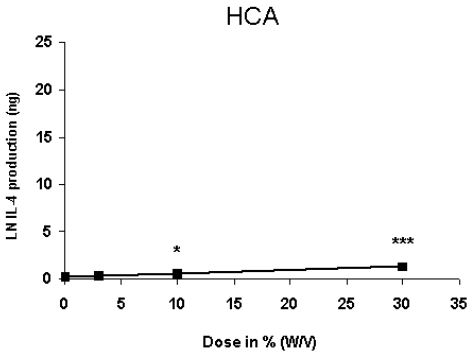
HCA
LN IL-4 production (ng)
Dose in % (W/V)
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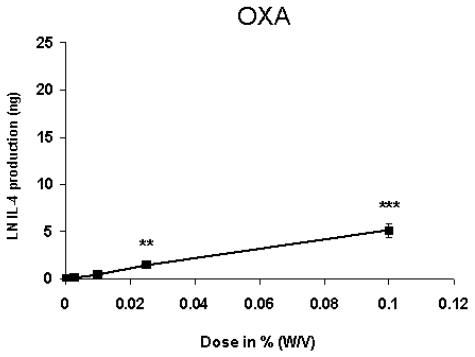
OXA
LN IL-4 production (ng)
Dose in % (W/V)
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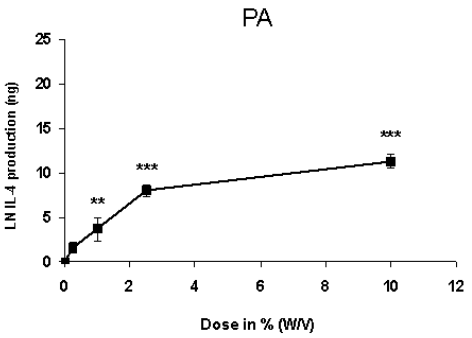
PA
LN IL-4 production (ng)
Dose in % (W/V)
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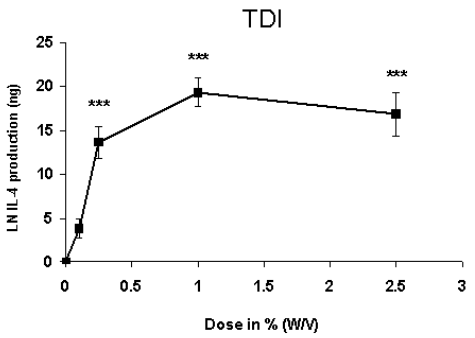
TDI
LN IL-4 production (ng)
Dose in % (W/V)
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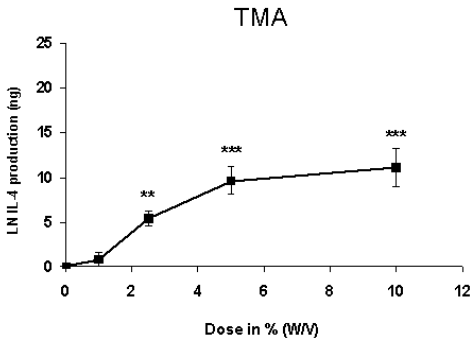
TMA
LN IL-4 production (ng)
Dose in % (W/V)
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RESULTS

Lymphocyte proliferation

Figure 1 shows the dose-response relationships for the proliferation of LN cells for the different chemicals. The concentrations used in the LLNA for DNCB, FITC, OXA, PA, TDI, and TMA are shown in Table 1. DNCB and TDI, induced significantly higher proliferative responses after topical application relative to vehicle treatment for the highest three concentrations used ($p < 0.001$, except 0.25% where $p < 0.01$). PA induced significantly higher proliferative responses for the three highest concentrations used ($p < 0.001$, except 1% where $p < 0.05$). FITC, OXA, and TMA induced significantly higher proliferative responses relative to vehicle treatment for the highest two concentrations used ($p < 0.001$, except 0.25% FITC where $p < 0.01$ and 0.025% OXA where $p < 0.05$). Only for the highest concentration of HCA, a statistically significant difference was found ($p < 0.001$). Application of DNCB, OXA, PA, and TMA resulted in similar dose-response curves for the LN cell proliferation. Application of TDI resulted in a higher maximal proliferative response, compared to the other compounds. The lowest proliferative responses were seen for FITC and HCA.

Cytokine production

We have measured cytokine dose-responses induced by the contact sensitizers DNCB, HCA, and OXA and the respiratory allergens FITC, PA, TDI, and TMA, at the same concentrations as were tested for proliferation. All chemicals showed a clear dose-response relationship for the three cytokines tested, IL-4, IL-10, and IFN-γ (Fig. 2, 3, and 4, respectively), except HCA that showed no dose-response relationship for IL-10. FITC, PA, TDI and TMA induced significantly higher IL-4 production after topical application relative to vehicle treatment ($p < 0.001$, except 1% PA and 2.5% TMA where $p < 0.01$, and 0.1% FITC where $p < 0.05$), with the exception of the lowest concentration. DNCB, HCA and OXA showed significant IL-4 production only at the highest two concentrations ($p < 0.001$, except 10% HCA where $p < 0.05$ and 0.025% OXA where $p < 0.01$). For the different chemicals, IL-10 production was rather similar compared to IL-4 production (Fig. 3). DNCB, PA, TDI, and TMA induced significant IL-10 production at the highest three concentrations ($p < 0.001$, except 2.5% TMA where $p < 0.01$, and 0.5% DNCB and 1 % PA, where $p < 0.05$). For FITC and OXA, only the highest concentration induced significantly higher IL-10 production relative to vehicle treatment ($p < 0.001$). HCA showed no statistically significant increase of IL-10 production. The LN cells derived from vehicle (AOO)-treated mice failed to produce detectable levels of IL-4 and IL-10. For FITC, HCA, PA, and TMA, IFN-γ production showed significant production only at the highest two concentrations ($p < 0.001$, except 0.25% FITC were $p < 0.01$, 2.5% PA and 10% HCA where $p < 0.05$). DNCB induced significantly higher IFN-γ production after topical application relative to vehicle treatment (0.25% where $p < 0.01$; and 0.5% and 1% where $p < 0.001$). TDI showed significant IFN-γ levels even at the lowest concentration ($p < 0.001$, except 0.1% where $p < 0.05$). OXA showed significant IFN-γ production only at the highest concentration ($p < 0.001$).

Generally, topical application of TDI resulted in the highest IL-4 production (Fig. 2). PA induced IL-4 similar to TMA. For PA, TDI, and TMA, topical application strongly induced IL-4 production, even at very low concentrations. DNCB and HCA induced much lower amounts of IL-4 compared to the respiratory allergens. At the highest concentrations, DNCB induced 2- to 3-fold less IL-4, while HCA induced 4- to 5-fold less IL-4 compared to the respiratory allergens. OXA showed similar IL-4 production compared to FITC. PA induced IL-10 similar to TMA, and the induction by DNCB, OXA, and FITC was about 5-fold lower than by PA and TMA (Fig. 3). Only TDI, showed a very high IL-10 production compared to DNCB and OXA (14-fold), FITC (11-fold), and PA and TMA (2- to 3-fold). The induction of IFN-γ was strongest for DNCB

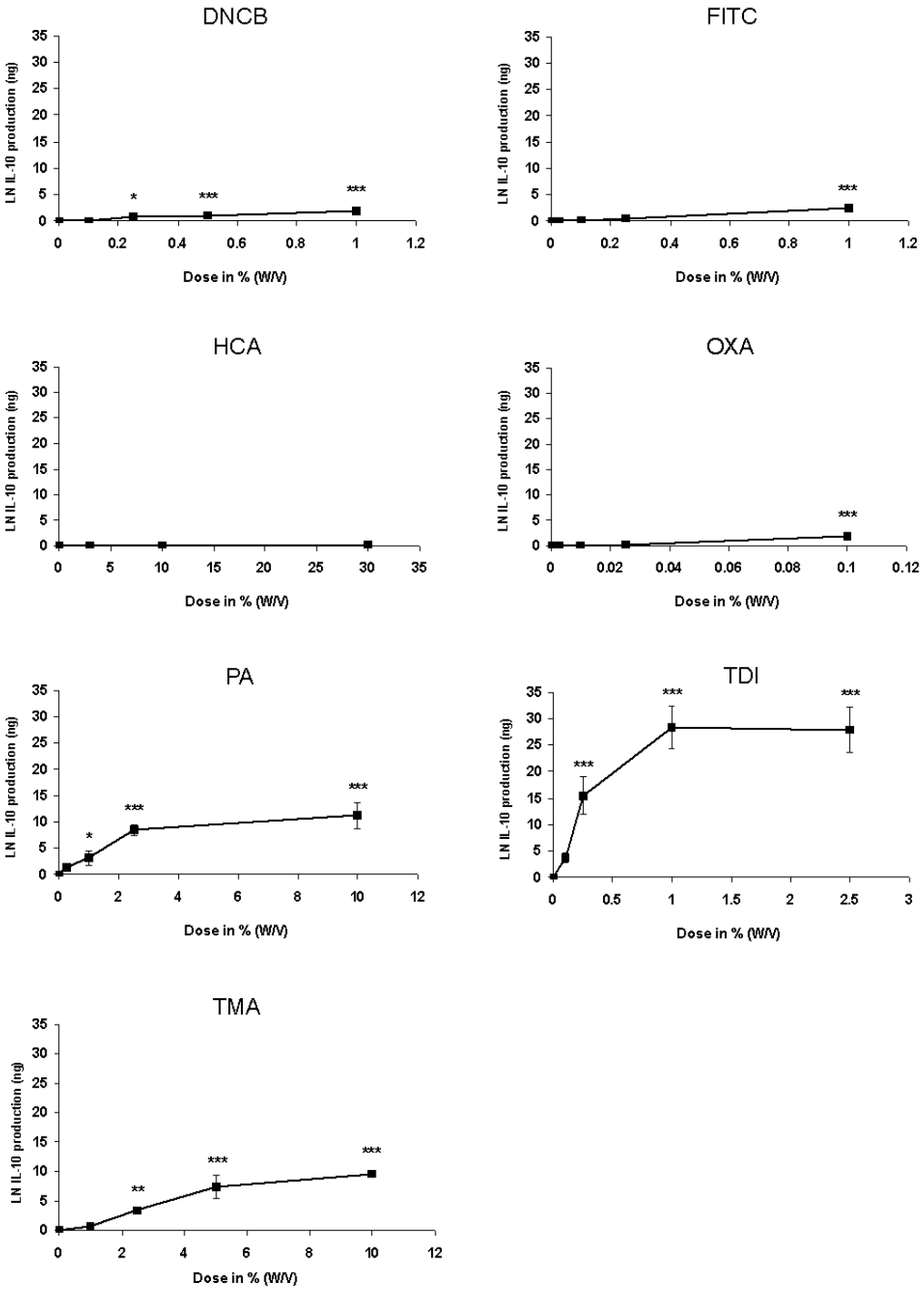


Figure 3
Effect of topical application on IL-10 production. Results are calculated by multiplying the IL-10 concentration by the LN cell number, and expressed as amount of IL-10 (ng) ± SEM (n=4). (*), (**), and (***), significantly different from AOO ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively).

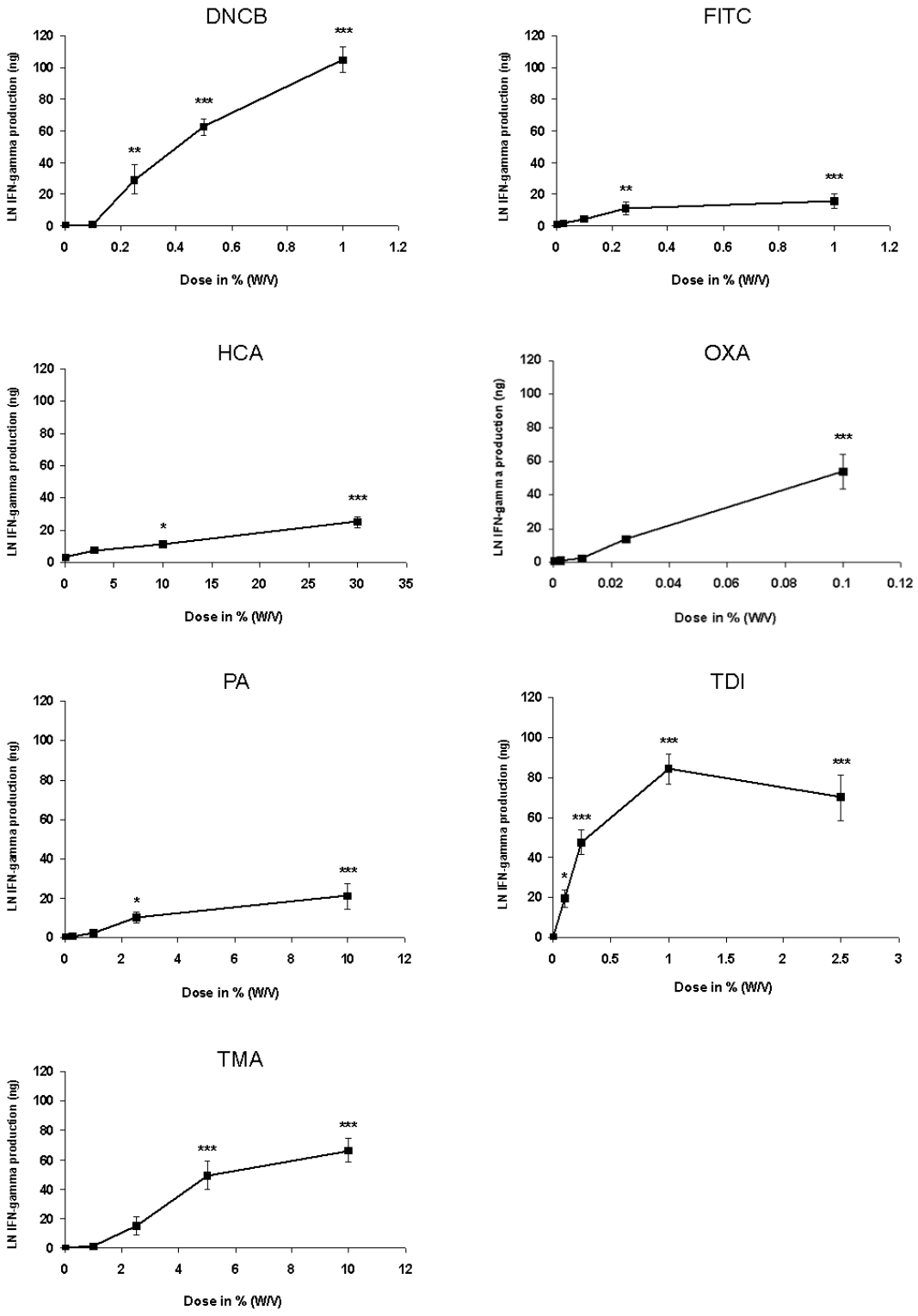


Figure 4
Effect of topical application on IFN-γ production. Results are calculated by multiplying the IFN-γ concentration by the LN cell number, and expressed as amount of IFN-γ (ng) ± SEM (n=4). (*), (**), and (***), significantly different from AOO ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively).

compared to the other six chemicals (Fig. 4). Even TDI, which showed a 2-fold higher proliferative response compared to the other chemicals (Fig. 1), showed lower IFN-γ production compared to DNCB. Similar responses were seen for OXA and TMA, while the induction of IFN-γ by FITC, HCA and PA was 2- to 4- fold lower compared to the other chemicals tested.

Cytokine production as a function of proliferative response

At several stimulation indices (SI's), the corresponding effective concentrations (EC) were calculated. For these EC-values, the corresponding IL-4, IL-10, and IFN-γ production was determined for the different chemicals (Tables 2, 3, and 4, respectively). Next, the IL-4, IL-10, and IFN-γ production was plotted against the stimulation indices (SI's), based on corresponding EC values (Fig. 5A, B, and C, respectively). HCA showed a maximal SI of 7, FITC of 20, PA of 30, and OXA of 50, compared to vehicle treated animals. For DNCB, TDI, and TMA, a maximal SI of 70 was used in the evaluation of the data. Even at low stimulation indices, clear differences in IL-4 and IL-10 production were seen between the contact sensitizers DNCB, HCA, OXA and the respiratory allergens FITC, PA, TDI, and TMA. At a SI=7, HCA induced lower amounts of IL-4 compared to FITC (1.6-fold), PA (3.1-fold), TDI (2.4-fold), and TMA (1.6-fold). Because no induction of IL-10 was found after exposure to HCA, EC values could not be calculated for this cytokine. OXA showed even greater differences in IL-4 production at a SI=7 compared to FITC (2.6-fold), PA (5.3-fold), TDI (4.1-fold) and TMA (2.8-fold). At a SI=10 very large differences were seen for FITC (41-fold), PA (83-fold), TDI (58-fold), and TMA (45-fold) compared to DNCB. At a SI=20, for FITC a 6.7-fold increase and for PA a 14.8-fold increase in IL-4 production was seen compared to DNCB. TDI (6.8-fold) and TMA (3.8-fold), showed increased IL-4 production at the maximal SI=70 (Fig. 5A) compared to DNCB. Similar IL-10 production were seen for FITC (5.5 fold at a SI=20), PA (6.4-fold at a SI=30), TDI and TMA (18.7-fold and 5.1-fold at a SI=70, respectively) compared to DNCB (Fig. 5B). OXA showed a similar IL-10 production as DNCB. TDI induced higher amounts of IL-10 production compared to the other six allergens, including the respiratory allergens FITC, PA, and TMA. TDI showed a 3- to 4-fold difference from a SI=30 and higher compared to TMA. At a SI=20, FITC induced higher amounts of IL-10 compared to the contact allergen OXA (3.9-fold). At a SI=7, HCA induced considerably higher IFN-γ production compared to FITC, PA and TMA (3.2-fold, 6.4-fold and 4.5-fold, respectively). At a SI=5 lower IFN-γ production was found for DNCB and OXA compared to the other allergens, whereas higher IFN-γ production was observed from a SI=35 and higher (Fig. 5C) for both contact allergens. Again, TDI induced higher amounts of IFN-γ production compared to the respiratory allergens FITC, PA and TMA (2.2-fold, 2.7-fold and 2.6-fold at SI=20, respectively).

DISCUSSION

The results presented in this paper, suggest that by directly linking the cytokine production to proliferation of the same cells, one can simultaneously identify, classify (based on lymphocyte proliferation), and characterize (based on cytokine production) chemicals in one single test. This approach is able to discriminate between respiratory and contact allergens independently of the choice of the concentrations used.

For respiratory allergens production of IL-4, and to a lesser extent IL-10, were discriminating already at low SI levels. For DNCB and OXA, even at a SI=3 much lower IL-4 production was seen compared to FITC, PA, TDI, and TMA. At a SI=20 a 6.7–fold lower IL-4 induction by DNCB compared to FITC was still seen, while even higher differences were seen compared to PA (14.8-fold), TDI (10.8-fold), and TMA (9.1-fold).

Table 2
Stimulation indices with the corresponding estimated concentration (EC, in %), and IL-4 production (in pg/ml) for the various chemicals. The models chosen for proliferation and IL-4 production are indicated between parentheses.

	DNCB		**FITC**		**HCA**		**OXA**		**PA**		**TDI**		**TMA**	
SI	EC (5)	IL-4 (5)	EC (4)	IL-4 (4)	EC (3)	IL-4 (2)	EC (5)	IL-4(5)	EC (4)	IL-4 (4)	EC (5)	IL-4 (4)	EC (5)	IL-4 (5)
3	0.09	ND	0.05	770	12.14	475	0.007	227	0.36	1549	0.03	1488	1.08	734
5	0.12	ND	0.11	1470	21.84	858	0.012	506	0.74	2948	0.05	2413	1.41	1454
7	0.14	20	0.17	2098	29.27	1351	0.016	793	1.15	4226	0.07	3290	1.67	2185
10	0.17	71	0.28	2909	x	x	0.022	1232	1.82	5892	0.09	4115	1.96	3180
20	0.24	650	0.99	4462	x	x	0.042	2565	4.88	9588	0.17	6999	2.69	5893
30	0.29	1664	x	x	x	x	0.064	3519	11.98	10920	0.25	9304	3.26	7672
50	0.41	2527	x	x	x	x	0.145	4167	x	x	0.43	12942	4.30	9282
70	0.61	2527	x	x	x	x	x	x	x	x	0.93	17129	5.54	9589
80	x	x	x	x	x	x	x	x	x	x	x	x	6.56	9600

ND, not detectable

Table 3
Stimulation indices with the corresponding estimated concentration (EC, in %), and IL-10 production (in pg/ml) for the various chemicals. The models chosen for proliferation and IL-10 production are indicated between parentheses.

	DNCB		**FITC**		**HCA**		**OXA**		**PA**		**TDI**		**TMA**	
SI	EC (5)	IL-10 (5)	EC (4)	IL-10 (5)	EC (3)	IL-10 (-)	EC (5)	IL-10 (5)	EC (4)	IL-10 (4)	EC (5)	IL-10 (5)	EC (5)	IL-10 (6)
3	0.09	ND	0.05	75	12.14	ND	0.007	2	0.36	1529	0.03	367	1.08	723
5	0.12	ND	0.11	225	21.84	ND	0.012	10	0.74	2875	0.05	958	1.41	1221
7	0.14	2	0.17	432	29.27	ND	0.016	28	1.15	4132	0.07	1744	1.67	1650
10	0.17	3	0.28	830	x	x	0.022	83	1.82	5571	0.09	2823	1.96	2221
20	0.24	427	0.99	2353	x	x	0.042	609	4.88	8557	0.17	8516	2.69	3650
30	0.29	1459	x	x	x	x	0.064	1503	11.98	9380	0.25	14552	3.26	4695
50	0.41	1464	x	x	x	x	0.145	1852	x	x	0.43	24110	4.30	6263
70	0.61	1464	x	x	x	x	x	x	x	x	0.93	27379	5.54	7564
80	x	x	x	x	x	x	x	x	x	x	x	x	6.56	8286

ND, not detectable

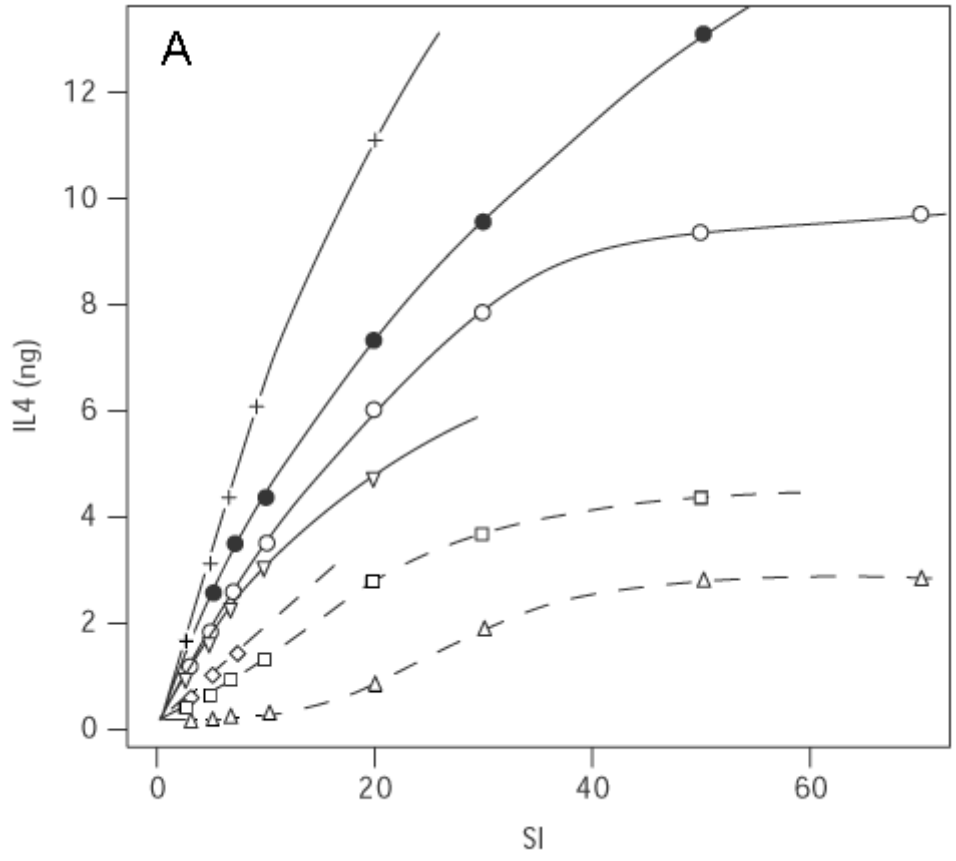
Table 4
Stimulation indices with the corresponding estimated concentration (EC, in %), and IFN-γ production (in pg/ml) for the various chemicals. The models chosen for proliferation and IFN-γ production are indicated between parentheses.

	DNCB		**FITC**		**HCA**		**OXA**		**PA**		**TDI**		**TMA**	
SI	EC (5)	IFN-γ (5)	EC (4)	IFN-γ (5)	EC (3)	IFN-γ (3)	EC (5)	IFN-γ (5)	EC (4)	IFN-γ (6)	EC (5)	IFN-γ (4)	EC (5)	IFN-γ (3)
3	0.09	263	0.05	2674	12.14	13096	0.007	1771	0.36	32	0.03	7486	1.08	2778
5	0.12	999	0.11	4938	21.84	18597	0.012	3812	0.74	710	0.05	11878	1.41	3957
7	0.14	3000	0.17	7118	29.27	22739	0.016	5923	1.15	3560	0.07	15848	1.67	5054
10	0.17	4959	0.28	9690	x	x	0.022	9272	1.82	8780	0.09	19429	1.96	6281
20	0.24	22187	0.99	14038	x	x	0.042	21352	4.88	11349	0.17	30614	2.69	11669
30	0.29	44513	x	x	x	x	0.064	34582	11.98	11394	0.25	38058	3.26	13584
50	0.41	86139	x	x	x	x	0.145	64238	x	x	0.43	46946	4.30	21331
70	0.61	88808	x	x	x	x	x	x	x	x	0.93	52408	5.54	32914
80	x	x	x	x	x	x	x	x	x	x	x	x	6.56	44463

Relative to DNCB, OXA showed smaller differences in IL-4 induction compared to FITC (1.7-fold), PA (3.7-fold), TDI (2.7-fold), and TMA (2.3-fold) (see Table 2). Both contact allergens DNCB and OXA showed even greater differences in IL-10 production compared to the respiratory allergens (see Table 3). In the smaller range of proliferation for HCA, IL-4 production suggests it being a respiratory allergen but the absence of IL-10 production and the high IFN-γ production suggests it to be a contact allergen. Notably, in contrast to DNCB and OXA, HCA showed no induction of IL-10, while at a SI=7 higher IFN-γ production was seen compared to PA and TMA (6.4-fold and 4.5-fold, respectively). From a SI≥20 DNCB and OXA showed higher IFN-γ production compared to the respiratory allergens FITC, PA, and TMA. TDI induced a higher IFN-γ production for 3<SI<35 compared to DNCB and OXA, and lower IFN-γ production from SI>35. Moreover, TDI showed a much higher IFN-γ production compared to the other respiratory allergens FITC, PA, and TMA (2.2-fold, 2.7-fold, and 2.6-fold at SI=20, respectively). IL-10 production from SI≥ 30 was considerable higher after application of TDI compared to all other allergens.

DNCB is a potent contact allergen known to cause allergic contact dermatitis in man (Kligman and Epstein, 1959), but is considered not to cause respiratory allergy (Botham *et al*., 1989). HCA is a contact allergen of mild to moderate potency (Dearman *et al*., 1998) and one of the chemicals that are recommended by the Organization for Economic Cooperation and Development (OECD, 1992) for use as a positive control in skin sensitization testing. Previous findings suggest OXA to be a potent contact allergen in both the GPMT (Gad *et al*., 1986) and the LLNA (Loveless *et al*., 1996). FITC was previously shown to be a contact sensitizer (Thomas *et al.*, 1980). Recent observations, however, identified FITC as a respiratory allergen based on a type 2 cytokine profile and specific IgE antibody response (Dearman and Kimber, 2000). PA, TDI, and TMA are all recognized occupational respiratory allergens (Hagmar *et al*., 1987; Murdoch and Pepys, 1987). However, PA and TMA can also penetrate the skin of humans and thus have the potential to elicit an allergic reaction in the skin (Bernstein *et al*., 1982). Contact sensitivity by TDI has also been demonstrated in humans (Malten, 1979) and animals (Tominaga *et al*., 1985; Scheerens *et al*., 1996). So, some compounds are capable in causing allergic reactions in either skin or respiratory tract and others in both. Our data indicate that the cytokine profile was clearly able to identify respiratory sensitizers, such as FITC, PA, and TMA and skin sensitizers, such as DNCB, HCA, and OXA. Somewhat different cytokine responses were seen after application of the contact allergen HCA compared to DNCB and OXA. HCA induced a considerably higher amount of IL-4 compared to DNCB, but still significant differences were seen compared to the respiratory allergens. Also higher amounts of IFN-γ were induced following application of HCA compared to DNCB and OXA, while no induction of IL-10 was observed. HCA can therefore be identified as a contact allergen. In addition, this result indicates that even at low levels of proliferation this distinction can be made.

Figure 5
Cytokine production as a function of the proliferative response. At certain stimulation indices (SI), the corresponding estimated concentrations were calculated (EC). Based on these EC-values, corresponding IL-4, IL-10 and IFN-γ production (A, B and C, respectively) was determined for DNCB (Δ), FITC (∇), HCA (◊), OXA ( ), PA (+), TDI (•), and TMA (o). The curves for the contact allergens are shown as dashed lines and the curves for the respiratory allergens as contineous lines.


A
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0
2
4
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8
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12
0
20
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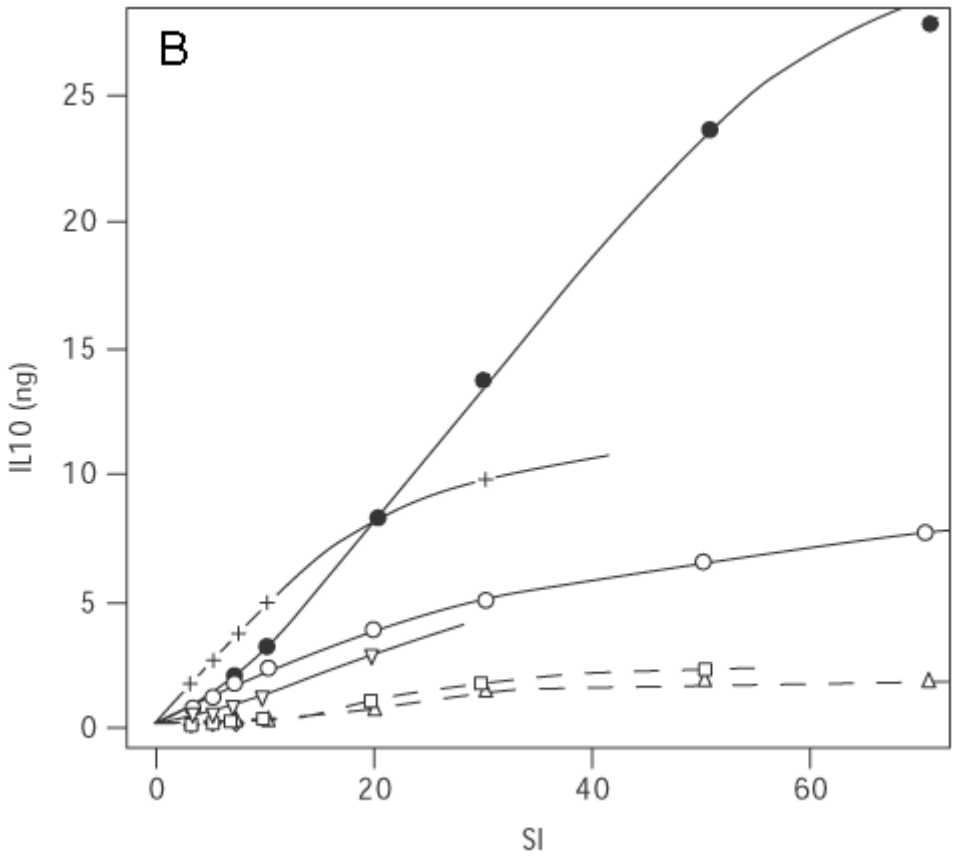
60
SI


B
IL10 (ng)
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5
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15
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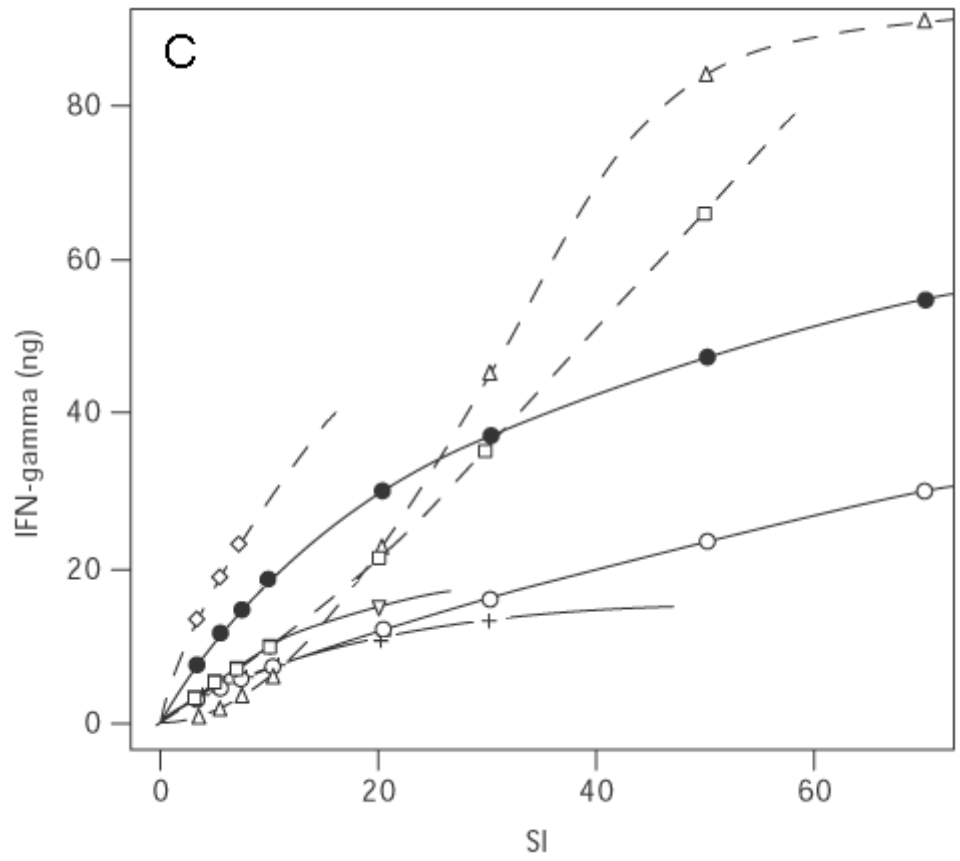
25
0
20
40
60
SI



TDI seems to be of a mixed nature, depending on the concentration used for sensitization. Remarkably, the fact that TDI besides considerable IL-4 production, also induces considerable amounts of IFN-γ may have been expected, because the compound has shown features of both contact and respiratory allergens in both humans and animals (Malten, 1979; Tominaga *et al*., 1985; Scheerens *et al*., 1996). In general, compounds that induce Th2 cells, mediate type I (immediate) hypersensitivity, and induce IgE and IgG1 production are respiratory sensitizers, and are associated with the production of high levels of IL-4 by the draining LN cells. However, skin sensitization with TDI can induce respiratory hypersensitivity with features of type IV hypersensitivity in mice (Scheerens *et al*., 1996). Also in humans, specific IgE is only detected in a minority of patients suffering from respiratory allergy induced by TDI (Karol, 1981; Karol *et al*., 1994). It may be postulated that TDI induces different types of responses, depending on the exposure concentrations. Our results are in line with recent observations by Manetz *et al*. (2001), who investigated IL-4 and IFN-γ mRNA levels of draining lymph node cells following dermal exposure to TDI, and found both to be increased.

Consequently, we propose that chemicals should be evaluated at concentrations that induce a similar induction of proliferation. When under these conditions a chemical induces a similar (or higher) IL-4 and IL-10 production compared to FITC, PA, TDI, and TMA, the sensitizer may be designated a respiratory sensitizer. A chemical may be identified as a contact allergen when IFN-γ production is similar to DNCB, HCA, and OXA while IL-4 production is similar to HCA, or IL-4 and IL-10 production are similar to DNCB and OXA. Importantly, these comparisons should be made on the entire dose response curve.

In conclusion, the aim of the present study was to develop a new approach to distinguish between contact and respiratory allergens, by measuring dose-dependent cytokine

profiles in a modified LLNA. For a range of concentrations, both proliferative responses and cytokine production were established. Estimated concentrations were derived at several stimulation indices (SI's). From the estimated concentrations, IFN-γ, IL-4, and IL-10 production as a function of stimulation indices were plotted. The data showed that FITC, PA, TDI, and TMA can be discriminated from DNCB, HCA, and OXA on the basis of both IL-4 and IL-10 production. A positive identification of the contact allergens on the basis of IFN-γ production, was observed only at very high stimulation indices (SI≥35) for DNCB and OXA, and already at low SI's (SI≤7) for HCA. On the basis of IL-10 and IFN-γ production, TDI showed different responses compared to FITC, PA, and TMA. By directly linking the cytokine production to proliferation of the same cells, discrimination between respiratory and contact allergens can be done independently of the choice of the concentrations used.

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CHAPTER 7

MODELLING PHYSICO-CHEMICAL PROPERTIES OF LOW MOLECULAR WEIGHT CHEMICALS FOR PREDICTION OF ALLERGENIC POTENCY

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In the present study we sought to predict the allergenic potency of chemicals solely on the basis of their molecular features. We calculated the physico-chemical properties of ten allergens (ethyl-p-aminobenzoate (BENZ), diethylamine (DEA), 2,4- dinitrochlorobenzene (DNCB), hexyl cinnamic aldehyde (HCA), 2- mercaptobenzothiazole (MBT), 4-ethoxymethylene 2- phenyloxazol-5-one (OXA), phthalic anhydride (PA), toluene diisocyanate (TDI), trimellitic anhydride (TMA), and tetramethyl thiuram disulfide (TMTD). Multiple regression analysis of the EC_3 (estimated concentration in % required for stimulation index of three in LLNA) against the different molecular features resulted in different models. EC_3 values formerly obtained with a modified LLNA were used as reference. For each model the predicted sensitizing values (PSVs) were calculated for the different chemicals and compared to the LLNA data. The weak allergens BENZ and DEA showed a PSV > 19, whereas a PSV < 5 was observed for the strong allergens DNCB, PA, TDI, TMA, and TMTD. Only the potent allergen OXA, together with the moderate allergens HCA and MBT, showed PSVs between 5 and 19. Based on the outcome, a scaling of chemicals can be suggested. For instance, if the PSV of an allergen is < 5, it is likely to be a potent allergen, between 5 and 10 a moderate allergen, and if an allergen has a PSV > 10 it is most likely to be a weak allergen. Because only a limited number of allergens was evaluated, the approach taken in this study can only be seen as a first step in the development of a model. Extension to a substantially larger dataset is neccessary in order to obtain a more reliable model. If succesfull, this approach may be used as a prescreen for the allergenic potency of chemicals and such an in silico approach may ultimately lead to a reduction in the use of experimental animals.

INTRODUCTION

Identification of contact allergens (skin sensitizers) is generally performed using animal models. The tests most commonly used are the guinea pig maximization test (GPMT) and the murine local lymph node assay (LLNA). The LLNA (Kimber et al., 1986; Kimber and Weisenberger, 1989) identifies the sensitizing capacity of low molecular weight chemicals as a function of induced proliferative responses in lymph nodes draining the site of application. For risk analysis an important next step is the determination of the sensitizing potency of allergens. Besides others (Basketter et al., 1999), we have previously described relative potency determination using a modified LLNA (Van Och et al., 2000; De Jong et al., 2002).

One approach to predict the sensitizing capacity, or even the potency, without the use of animals is the use of computer-based models (expert systems). These models link physico-chemical properties of a range of compounds with human clinical data or experimental animal data to identify structural alerts for skin sensitization. The characteristics of a chemical are generally thought to be determined by its atomic composition and structure. Especially physico-chemical properties are thought to predict a large portion of a molecule's behaviour. Several studies have been described in which the sensitizing potential of chemicals together with certain physical and chemical parameters of these compounds were used to build a mathematical model (Magee et al., 1994; Graham et al., 1996; Hostynek and Magee, 1997). Examples of parameters used in these models are parameters for reactivity (water solubility, molecular weight), and penetration (molecular refraction, log P (octanol/water partition coefficient)).

Barratt et al. (1994) reported the as yet greatest success in predicting the activity of contact sensitizers, notably a sensitivity rate of 98%. They used a knowledge-based computer system called DEREK ("Deductive Estimation of Risk from Existing Knowledge"; Sanderson and Earnshaw, 1991). DEREK embodies both a controlling program and a chemical rulebase and is originally based on an Unilever database containing the results of 294 defined substances in the GPMT. This rulebase contains a subset of approximately 50 rules that describes chemical substructures known or believed to be responsible for the chemical reactivity component of skin sensitization. Moreover, Barratt (1996) developed a mathematical model to calculate the theoretical irritation potential of chemicals. In this model the predicted irritation index (PII), which is a measure for erythema and oedema occurring during the first 3 days of exposure to the chemical, was predicted using the octanol-water partition coëfficient (logP) and the molecular volume (MV) as markers for skin permeability, and the dipole moment (dipole) as a marker for chemical reactivity.

In the present study we sought to estimate the *sensitizing* potency of allergens solely based on their physico-chemical properties. Besides the properties mentioned above we included five properties in our investigation, i.e. octanol-water partition coefficient (logP), molecular volume (MV), melting point (mpt), adsorption coefficient (AC), molecular weight (MW), and surface. These parameters are supposed to indicate the accessibility of the chemicals to the cells. Dipole moment, aqueous solubility (AS), and ionisation constant (pKa) are supposed to indicate the reactivity of the allergen. Because the LLNA is able to provide quantitative data on the sensitizing potency, much more than the GPMT, we used the data we previously obtained with a modified LLNA (Van Och et al., 2000) as reference. Stepwise regression analysis resulted in simple mathematical models described as equations. Predicted sensitizing values (PSVs) were calculated for the different chemicals and the potency of the sensitizing capacity was compared to the classification we previously found in a modified LLNA (Van Och et al., 2000).

MATERIALS AND METHODS

Chemicals

Benzocaine (BENZ, ethyl-p-aminobenzoate; 99% purity; Sigma-Aldrich Chemie B.V., Zwijndrecht, NL, CAS 94-09-7), DEA (diethylamine; 99.5%; free base solution; Sigma-Aldrich, CAS 109-89-7), DNCB (2,4-dinitrochlorobenzene; 98%; Sigma-Aldrich, CAS 97-00-7), HCA (hexyl cinnamic aldehyde; 85%; Sigma-Aldrich, CAS 101-86-0), MBT (2-mercaptobenzothiazole; 98%; Sigma-Aldrich, CAS 149-30-4), oxazolone (OXA, 4-ethoxymethylene 2- phenyloxazol-5-one; 90%; Sigma-Aldrich, CAS 15646-46-5), PA (phthalic anhydride; 99%; Sigma-Aldrich, CAS 85-44-9), TDI (toluene 2,4-diisocyanate; 99.8%; Sigma-Aldrich, CAS 584-84-9), TMA (trimellitic anhydride; 1, 2, 4- benzenetricarboxylic anhydride; 97%; Sigma-Aldrich, CAS 552-30-7), TMTD (tetramethylthiuramdisulfide; 98%; Sigma-Aldrich, CAS 137-26-8) were tested.

Calculation of physico-chemical properties

For the estimation of physico-chemical properties of the different chemicals, the chemical structures were constructed using ChemSketch software from ACD labs (version 5.0 for Microsoft Windows, Advanced Chemistry Development Inc., Ontario, Canada). Dipole moments were calculated using MOPAC software (version 6.0, Fujitsu Systems, Amsterdam, NL). Surface areas were calculated from the same structures using PCMODEL software (version 4.0, Serena Software, CA, USA), with the spacing increment set at 0.25 Å. For the calculation of structural properties, the structures were optimised according to the PM3 method (Stewart, 1989). Molecular volume (MV) was calculated from the surface under the assumption that the molecule is spherically shaped. Values for octanol-water partition coëfficient (logP), adsorption coefficient (AC), aqueous solubility (AS), and molecular weight (MW) were calculated using the ACD/I-Lab Web service (Advanced Chemistry Development Inc., Ontario, Canada, http://www2.acdlabs.com/ilab/). Melting points (mpt) were obtained from catalogues of commercial suppliers. The calculated physical and chemical properties are shown in Table 1.

Correlation/multiple regression analysis

Correlation of the different properties with the EC3 values in the LLNA were derived with Mathematica version 4.0 (Wolfram Software, Oxfordshire, UK) and are summerized in a correlation matrix (Table 2).

The different models were generated by stepwise regression analysis using Mathematica version 4.0.

RESULTS

Calculation of the physico-chemical properties

In order to predict the behaviour of the chemicals used in this study, several of their physico-chemical properties were calculated. These properties are given in table 1.

The ionisation constant (pKa), formerly used as a measure of cytotoxicity (Barratt, 1998), could not be calculated for DNCB, HCA, PA, and TDI because these molecules contained unsupported atoms according to the ACD/I-Lab software program. Therefore, the parameter pKa was not used in further analysis.

Table 1
Physico-chemical properties of the chemicals analysed. Values are shown as indicated by the manufacturer, or found in chemical databases, or as calculated using chemical modelling software (see Materials and Methods for details). Indicated in the table are molecular weight (MW), melting point (Mpt), octanol-water partition coëfficient (LogP), dipole moment, surface area, molecular volume (MV), adsorption coefficient (AC), and aqueous solubility (AS). The different properties (P_x) are numbered for use in mathematical modelling.

	P_1	P_2	P_3	P_4	P_5	P_6	P_7	P_8
Chemical	MW (Dalton)	Mpt (Celsius)	LogP	Dipole (Debyes)	Surface	MV ($Å^3$)	AC	AS (mol/L)
BENZ	165.19	90	1.80	2.96	170	210	2.7	4.4e-3
DEA	73.14	-50	0.63	1.79	129	138	1.0	1.7
DNCB	202.50	53	2.06	4.94	170	210	2.5	4.4e-4
HCA	216.32	39	5.33	2.25	201	268	4.3	4.3e-6
MBT	167.25	180	2.38	5.81	146	165	0.2	5.4e-2
OXA	217.22	96	2.22	2.67	211	288	2.6	2.4e-3
PA	148.12	133	1.12	5.67	138	152	2.0	6.8e-2
TDI	174.16	13	2.32	1.72	67	52	2.6	0.2
TMA	192.13	165	1.07	5.04	27	13	1.0	8.1e-3
TMTD	240.41	155	1.31	5.90	233	334	2.3	9.2e-4

Correlation factors

The correlation factors between the different properties and the correlation factors of the different properties with the reference (EC_3 value in the LLNA) were calculated, and are shown as a correlation matrix (Table 2). The correlation factors range from −1 (100% negative correlation) to 1 (100% positive correlation). A correlation factor of zero indicates no correlation between two variables. Molecular volume (MV) and surface show a +0.992 correlation, with MV being calculated from the surface. Negative correlations were found for AC, dipole moment, LogP, MP, MV, and MW compared to the reference parameter EC_3, whereas AS and surface show a positive correlation. Aqueous solubility (AS) shows the highest correlation with EC_3 (+0.806), whereas the lowest correlation was found for MV (-0.025).

Table 2
Correlation matrix. The correlation factors between the different properties and the correlation factors of the different properties with the reference (EC_3 value in the LLNA) were calculated.

	MW	Mpt	LogP	Dipole	Surface	MV	AC	AS	EC_3
MW	1	0.488	0.449	0.303	0.406	0.493	0.492	-0.826	-0.748
Mpt	0.488	1	-0.139	0.829	0.0384	0.0861	-0.330	-0.680	-0.569
LogP	0.449	-0.139	1	-0.320	0.331	0.333	0.682	-0.379	-0.114
Dipole	0.303	0.829	-0.320	1	0.065	0.084	-0.432	-0.450	-0.489
Surface	0.406	0.0384	0.331	0.065	1	0.992	0.462	-0.172	0.035
MV	0.493	0.0861	0.333	0.084	0.992	1	0.482	-0.222	-0.025
AC	0.492	-0.330	0.682	-0.432	0.462	0.482	1	-0.351	-0.174
AS	-0.826	-0.680	-0.379	-0.450	-0.172	-0.222	-0.351	1	0.806
EC_3	-0.748	-0.569	-0.114	-0.489	0.035	-0.025	-0.174	0.806	1

Stepwise regression analysis

In order to estimate the contribution of each physico-chemical property to the empirical endpoint, stepwise regression analysis was performed.

The different properties as shown in Table 1 are indicated by corresponding P numbers, such as P_1 for molecular weight, P_2 for melting point, P_3 for logP etc.

Step 1:
{P_1, P_2, P_3, P_4, P_5, P_6, P_7, P_8}
[Partial Sum of Squares {265.64, 125.25, 225.42, 50.10, 201.98, 208.24, 140.28, 133.52}]
P_4 (dipole) was removed (value 50.10).

Step 2:
{P_1, P_2, P_3, P_5, P_6, P_7, P_8}
[Partial Sum of Squares {284.07, 86.41, 207.34, 192.53, 200.16, 90.32, 87.03}]
P_2 (MP) was removed (value 86.41).

Step 3:
{P_1, P_3, P_5, P_6, P_7, P_8}
[Partial Sum of Squares {239.44, 135.43, 121.15, 135.14, 5.15, 2.98}]
P_8 (AS) was removed (value 2.98).

Step 4:
{P_1, P_3, P_5, P_6, P_7}
[Partial Sum of Squares {1016.73, 134.99, 191.88, 227.18, 4.73}]
P_7 (AS) was removed (value 4.73).

Step 5:
{P_1, P_3, P_5, P_6}
[Partial Sum of Squares {1017.16, 161.25, 187.40, 222.45}]
P_3 (logP) was removed (value 161.25).

Step 6:
{P_1, P_5, P_6}
[Partial Sum of Squares {877.24, 85.56, 114.73}]
P_5 (surface) was removed (value 85.56).

Step 7:
{P_1, P_6}
[Partial Sum of Squares {1134.24, 247.92}]
P_6 (MV) was removed (value 247.92).

For each step described above a corresponding model was generated to describe the predicted sensitizing value (PSV):

Model 1:

PSV_1 =385.19 – 1.32MW – 0.45mpt + 9.69LogP + 4.30Dipole – 3.79Surface + 2.66MV – 18.90AC – 84.61AS
$n = 10$; $r^2 = 0.96$

Model 2:

PSV_2 =252.70 – 0.88MW – 0.22mpt + 6.36LogP – 2.32Surface + 1.65MV – 11.86AC– 46.99AS
$n = 10$; $r^2 = 0.93$

Model 3:

$PSV_3 = 110.29 – 0.50MW + 4.70LogP – 1.03Surface + 0.74MV – 0.99AC – 2.96AS$

$n = 10$; $r^2 = 0.88$

Model 4:

$PSV_4 = 98.36 – 0.45MW + 4.51LogP – 0.92Surface + 0.66MV – 0.94AC$

$n = 10$; $r^2 = 0.87$

Model 5:

$PSV_5 = 97.32 – 0.45MW + 4.02LogP – 0.90Surface + 0.65MV$

$n = 10$; $r^2 = 0.87$

Model 6:

$PSV_6 = 77.38 – 0.36MW – 0.55P_5 + 0.43MV$

$n = 10$; $r^2 = 0.78$

Model 7:

$PSV_7 = 46.92 – 0.27MW + 0.059MV$

$n = 10$; $r^2 = 0.72$

The inclusion of more variables resulted in a refinement of the model (i.e. a further increase in r^2). Only the removal of the adsorption coefficiënt (AC) in the equation (from model 4 to model 5) did not result in an decrease of r^2. The PSVs for each chemical were calculated in the different models and are summerized in Table 3. The allergens BENZ, DEA, HCA, and MBT showed high similarity with the EC_3 values formerly obtained in model 4–7. These differences in potency were similar to the LLNA data. BENZ, DEA, and HCA showed a PSV > 10. A PSV < 5 was observed for the allergens DNCB, PA, TDI, TMA, and TMTD, whereas a PSV between 5 and 10 was observed for OXA and MBT.

DISCUSSION

In the present study we sought to determine the allergenic potency of chemicals solely on the basis of their physico-chemical properties. Using multiple regression analysis, various models, being linear equations with different number of parameters, were generated and predicted sensitising values (PSVs) were calculated for the different chemicals and compared to LLNA data formerly obtained by us (Van Och et al., 2000). Based on the quantitative data (Table 3) and the classification (Table 4), model 2 was chosen for further evaluation. Since the dipole moment is not included in model 2, it is of little value to the specific model. This model was used to calculate the predicted sensitizing value (PSV). The weak allergens BENZ and DEA (EC_3 > 20% in the LLNA), showed PSVs > 19 in model 1 and 2. HCA and MBT, known as mild to moderate allergens (5 < EC_3 < 18 in the LLNA), showed PSVs between 9 and 15. The more potent allergens DNCB, PA, TDI, TMA, and TMTD (EC_3 < 1% in the LLNA), showed PSVs below 5. Only OXA, the most potent allergen in the LLNA, showed a higher PSV compared to the other strong allergens. Based on these findings, a scaling of chemicals can be suggested. For instance, if the PSV of an allergen is < 5, it is likely to be a potent allergen, between 5 and 10 a moderate allergen, and if an allergen has a PSV > 10 it is most likely to be a weak allergen.

The different chemicals can now be ranked based on the derived PSVs presented in this paper. The predicted classification proved to be promising. It showed similar ranking for the allergens BENZ, DEA, HCA, and MBT compared to the classification found in the LLNA. These allergens showed no overlap of the 90%-confidence intervals in the LLNA. The more potent allergens showed variable responses. The most plausible explanation for this

discrepancy is that these allergens show an EC_3 level in the same range. DNCB, OXA, PA, TDI, TMA, and TMTD showed EC_3 values between 0.044% and 0.66% with overlapping 90%-confidence intervals in the LLNA (Table 3).

Table 3
Predicted sensitizing values (PSVs) of the allergens in different models and the EC_3 values formerly obtained in a modified local lymph node assay (LLNA), together with their corresponding 90%-confidence intervals.

Chemical	Model 7	Model 6	Model 5	Model 4	Model 3	Model 2	Model 1	LLNA[a] EC_3 (%)[b]	L05-L95 [c]
BENZ	14.76	14.78	13.81	11.89	13.87	19.02	20.29	22.026	16.58-33.95
DEA	35.35	39.49	40.6	39.81	39.98	40.14	40.56	39.784	34.08-47.70
DNCB	4.63	1.28	-2.04	-3.62	-3.45	-1.63	2.92	0.044	0.025-0.078
HCA	4.41	4.31	14.85	13.11	14.37	12.82	13.24	17.689	15.08-20.85
MBT	11.56	7.91	7.59	8.34	9.34	9.89	13.11	9.669	8.02-12.19
OXA	5.32	7.05	5.89	4.23	5.32	9.27	5.77	0.013	0.004-0.025
PA	15.92	13.56	9.82	8.29	9.56	4.05	8.14	0.357	0.23-0.56
TDI	3.01	0.25	1.85	0.76	0.50	1.60	-2.13	0.109	0.048-0.26
TMA	-4.15	-1.00	-0.63	-0.41	0.12	0.81	2.82	0.218	0.13-0.41
TMTD	1.83	6.45	1.99	0.19	1.34	-1.01	-1.40	0.659	0.55-0.82

[a] Van Och et al., 2000
[b] estimated concentration in % required for SI=3
[c] 5th and 95th percentile

The scope of the investigation as presented in the present paper, was not the identification of allergens but to assess the potency of known allergens, by analyzing the molecular features of a chemical in order to predict its sensitizing potency.
For a low molecular weight chemical to act as skin sensitizer it is critical to penetrate the outer lipophilic layer of the skin (the stratum corneum) into the viable epidermis where it binds covalently to skin proteins thus forming an immunogenic complex (Landsteiner and Jacobs, 1936). The percutaneous adsorption of a chemical is a vital characteristic in the risk assessment process for chemicals that come into contact with the skin and is therefore a popular subject of research (Flynn, 1990; Potts and Guy, 1992; Barratt, 1994; 2000). Previously, Barratt (2000) showed regression analysis with the inclusion of the variables logP, molecular volume, and melting point. The melting point of a chemical, together with its lipophilicity, modeled by logP, has been shown to be an important determinant of its aqueous solubility (Suzuki, 1991). As a refinement of that approach we added molecular weight and adsorption coefficient as determinants important for accessibility to cells. Dipole moment, aqueous solubility, and pKa were chosen as indicators of the reactivity of a chemical. Dipole moment has previously been used succesfully, as a reactivity parameter in a QSAR study of the eye irritation potential (Barratt, 1995).

The chemicals presented in this paper, are all recognized allergens. In animal studies as well as from clinical data these chemicals have shown their ability to induce allergenic reactions in the skin. In a previous study, we quantified the sensitizing capacity of the chemicals used in the present study by dose-response modeling. At a stimulatory index of three, which is generally recognized as a threshold of sensitizing potency (Basketter et al., 1999), the corresponding estimated concentration was calculated (EC_3). Here, the order of the chemicals with increasing EC_3 values (and thus decreasing allergenic potency) was found to be: OXA < DNCB < TDI < TMA < PA < TMTD < MBT < HCA < BENZ < DEA (Van Och et al., 2000; Table 3).

Table 4
Ranking of the allergens in different models (decreasing potency from top to bottom), and classification of the allergens in a modified local lymph node assay (LLNA). The number of parameters are shown between parentheses.

Model 7 (2)	Model 6 (3)	Model 5 (4)	Model 4 (5)	Model 3 (6)	Model 2 (7)	Model 1 (8)	LLNA[a]
TMA	TMA	DNCB	DNCB	DNCB	DNCB	TDI	OXA
TMTD	TDI	TMA	TMA	TMA	TMTD	TMTD	DNCB
TDI	DNCB	TDI	TMTD	TDI	TMA	TMA	TDI
HCA	HCA	TMTD	TDI	TMTD	TDI	DNCB	TMA
DNCB	TMTD	OXA	OXA	OXA	PA	OXA	PA
OXA	OXA	MBT	PA	MBT	OXA	PA	TMTD
MBT	MBT	PA	MBT	PA	MBT	MBT	MBT
BENZ	PA	BENZ	BENZ	BENZ	HCA	HCA	HCA
PA	BENZ	HCA	HCA	HCA	BENZ	BENZ	BENZ
DEA	DEA	DEA	DEA	DEA	DEA	DEA	DEA

[a] Van Och et al. (2000)

The allergen ZDMC was not taken in the present study. According to the ACD/I-Lab software program, certain properties such as logP, AC, and AS, could not be calculated for a multicomponent structure (data not shown).

In conclusion, allergens (here low molecular weight chemicals) need certain characteristics that convey them with the potential to induce allergic sensitization. In the present study, we sought to develop a model with the incorporation of a variety of molecular features, being determinants important for permeability through the skin and reactivity in the skin. Multiple regression analysis of the EC_3 with the different properties resulted in different equations from which the PSV could be calculated. Potency evaluation resulted in a classification of the different chemicals, where the weak allergens could be distinguished from the strong allergens. Such an *in silico* approach may ultimately lead to a reduction in the use of experimental animals. Nevertheless, further validation with additional sensitizers, irritants and negative compounds is neccessary.

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CHAPTER 8

ASSESSMENT OF POTENCY OF ALLERGENIC ACTIVITY OF LOW MOLECULAR WEIGHT COMPOUNDS BASED ON IL-1α AND IL-18 PRODUCTION BY A MURINE KERATINOCYTE CELL LINE

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Screening for allergenic potency of low molecular weight compounds is generally performed using animal models. The tests most commonly used are the guinea pig maximization test and the murine local lymph node assay (LLNA). Over the last decade considerable progress has been made in unraveling the mechanisms of skin sensitization, including effects on the production of cytokines by the different cell types of the skin. This knowledge provides us with the opportunity to develop in vitro tests as an alternative to in vivo sensitization testing. In the present study we used a test system comprised of the murine epidermal keratinocyte cell line HEL30. We exposed this cell line to the allergen ethyl-p-aminobenzoate (benzocaine), diethylamine (DEA), 2,4- dinitrochlorobenzene (DNCB), and phthalic anhydride (PA). IL-1α and IL-18 dose-response data were evaluated by non-linear regression analysis, and at a certain ratio of cytokine production of treatment versus control, the corresponding allergen concentration was calculated. For both cytokines DNCB showed the strongest potency followed in this order by PA, benzocaine, and DEA. This classification was similar to our previous findings obtained in the LLNA. The approach taken in this study may be used as a screening method for the allergenic potency of chemicals, especially since the ranking of chemicals on the basis of allergenicity also proves to be possible in vitro.

INTRODUCTION

Predictive tests to identify sensitizing properties of chemicals are carried out on a large scale. The tests most commonly used are the guinea pig maximization test (Magnusson and Kligman, 1969) and the occluded patch test of Buehler (Buehler, 1965) in the guinea pig. More recently, alternative test methods have been described in the mouse, including the local lymph node assay (LLNA, Kimber *et al*, 1986; Kimber and Weisenberger, 1989) and the mouse ear swelling test (Gad *et al*, 1986). However, there is as yet, no accepted method for the identification *in vitro* of skin sensitizing chemicals.

Besides the identification of sensitizers, an important next step, is to establish the sensitizing potency of allergens. We have previously described relative potency determination with the LLNA (Van Och *et al*, 2000), where dose responses were evaluated by nonlinear regression analysis. Concentrations inducing a certain stimulation index (SI) were estimated based on a curve fitting method. Chemicals that elicit a SI of 3 (treated animals over vehicle control) or more in the LLNA are considered as being sensitizers. The concentrations inducing a SI=3 (EC_3) were used for the comparison of sensitizing potential.
In the present study we investigated the relative potency of known allergens *in vitro* based on cytokine dose-response relationships using the murine keratinocyte cell line HEL30. The anatomical location of keratinocytes and their significant role in the development of allergic contact dermatitis justifies the use of keratinocyte-derived cytokine production as read-out to evaluate sensitizing potency. Keratinocytes not only represent the first target for irritants (DeLeo *et al*, 1987) but may also act as a signal transducer, converting non-specific exogenous stimuli into the production of cytokines and chemotactic factors, and expression of adhesion molecules (Barker *et al*, 1991). In principle, a test system comprised of keratinocytes alone may not be useful in establishing allergenic potency as these cells lack antigen presenting capacity. In the epidermis this function is exerted by Langerhans cells (LC). Nevertheless, it has been hypothesized that the irritant capacity of allergens presents an additional risk factor (Cumberbatch *et al*, 1993; Grabbe *et al*, 1996) so that irritant allergens may be stronger allergens than non-irritant ones. This suggests that evaluating irritant potency of allergenic chemicals may be helpful to establish their allergenic potency. For this reason, it can be suggested that the potency of chemicals to induce cutaneous sensitization can be assessed as a function of keratinocyte cytokine expression, mediators produced by these cells, and adhesion molecule expression. *In vitro* responses of keratinocytes to sensitizing agents may therefore yield information that may be helpful in classification of the sensitizing potency of such chemicals.

All four chemicals tested in this paper have proven their sensitizing potency based on clinical data. 2,4- dinitrochlorobenzene (DNCB) is a potent contact allergen known to cause allergic contact dermatitis in man (Kligman and Epstein, 1959). Also ethyl-p-aminobenzoate (benzocaine; Cronin, 1980), diethylamine (DEA; Kaniwa *et al*, 1993) and phthalic anhydride (PA; Bernstein *et al*, 1982) have the potential to elicit an allergic reaction in human skin. Besides a skin allergen, PA is also known as an occupational respiratory allergen (Hagmar *et al*, 1987).
Epidermal keratinocytes are known to produce and excrete a wide range of cytokines (Haas *et al*, 1992). Cells were exposed to benzocaine, DEA, DNCB and PA, known allergens with different sensitizing potency (Van Och *et al*, 2000). The cytokine dose-response data were evaluated by non-linear regression analysis and at a stimulatory index (SI) of three the corresponding estimated concentration was calculated (EC_3). We used these EC_3 values to rank these chemicals and to compare the outcome of the present study with those obtained from the local lymph node assay (LLNA).

METHODS

Chemicals

Benzocaine (ethyl-p-aminobenzoate; 99% purity; Sigma-Aldrich Chemie B.V., Zwijndrecht NL), DEA (diethylamine; 99.5%; free base solution; Sigma-Aldrich), DNCB (2,4-dinitrochlorobenzene; 98%; Sigma-Aldrich), PA (phthalic anhydride; 99%; Sigma-Aldrich) were used. In the LLNA these chemicals were tested in 4:1 acetone/olive oil (AOO). For the in vitro analysis the chemicals were dissolved in ethanol. The final ethanol concentration was 1%.

Cell culture and treatment

The C3H mouse-derived keratinocyte cell line HEL30 (Fusenig *et al*, 1983, 1985) was kindly provided by Prof. N. Fusenig (German Cancer Institute, Heidelberg, Germany). HEL30 cells were cultured in Dulbecco`s modified Eagle`s medium (Gibco, Life Technologies, Breda, the Netherlands) supplemented with 10% fetal calf serum (PAA, Linz, Austria), penicillin-streptomycin (100 U/ml-0.1 mg/ml), and L-glutamine (2 mM). Cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 in air. The medium was changed twice a week and cultures were split by treating the cells with 0.25% trypsin (in PBS) for 5 min at 37°C before reaching confluency.

Cells were seeded in 12-well plates (Costar, Cambridge, MA) at a density of $1.5x10^5$/ml. After 24 hr the cells were washed once with phosphate buffered saline (PBS) and exposed to several concentrations of various sensitizers in culture medium per well. After 24 hr of incubation, supernatants were obtained and used for the determination of extracellular cytokine levels. The cells were then washed once with PBS and lysed with 0.5 ml 0.5% Triton X-100 (Merck, Amsterdam, NL) for 1 hr at 37°C. The cell lysate was used for determination of intracellular cytokine levels. In parallel cultures the cells were counted using a Coulter Counter (Z2, Coulter Electronics, Mijdrecht, NL). All tests were performed in triplicate. Samples were stored at -80°C until the assay was performed.

RNA isolation

Keratinocytes were harvested by trypsinization followed by centrifugation (10 minutes, 311g, 4°C), and were counted using a Coulter Counter. RNA was isolated using the SNAP total RNA isolation kit (Invitrogen, San Diego, CA) according to the manufacturer's instructions. All steps were performed at room temperature, except when indicated. Centrifugation steps were performed at 13.000 rpm. Briefly, 2 volumes (vol; 1 vol = 300 µl) of lysis buffer (5.3 M guanidine-HCl, 1.5% Triton-X100, 2.5 mM Tris-HCl, pH 7.5, 0.25 mM EDTA) was added to the KC. The KC were then homogenized using a pellet pestle, and 1 vol isopropanol was added. After mixing the sample was transferred to a column. The column was centrifuged for 1 min and 1 vol wash solution (100 mM NaCl) was added. The column was centrifuged again for 1 min and again 1 vol wash solution was added. The column was centrifuged for 2 min. After adding 0.225 vol water to the column and 5 min incubation, RNA was eluted by centrifugation for 1 min. After DNase treatment for 10 min at 37°C, 0.75 vol binding buffer (7M guanidine-HCl, 2% Triton-X100) was added and mixed. Then 0.5 vol isopropanol was added. After mixing the sample was transferred to a fresh column. The column was washed as described above. After adding 0.07 vol water to the column and 5 min incubation, RNA was eluted by centrifugation for 1 min. The RNA concentration was measured spectrophotometrically at 260 nm. RNA was subjected to electrophoresis on a 1 % agarose gel and visualized by ethidium bromide staining. RNA was used only when both 28 S rRNA and 18 S rRNA bands were intact. Samples were stored at -70 °C until use.

cDNA synthesis

One µg RNA was reverse transcribed using the Reverse Transcription System (Promega, Leiden, the Netherlands). Briefly, 10.5 µl water containing 1 µg RNA was mixed with 0.5 µl oligo(dT) primer (500 µg/ml) and incubated for 10 min at 70 °C. Samples were then incubated at 4 °C for at least 5 min. To the mixture, 4 µl $MgCl_2$ (25 mM), 2 µl 10*RT-buffer, 2 µl dNTP (10 mM each), 0.5 µl RNasin (50 U/µl) and 0.5 µl AMV-RT (30 U/µl) was added and incubated at 42 °C for 1 hr. The reaction was stopped by heating the samples for 5 min at 72 °C. Samples were stored at -20 °C until use.

Polymerase chain reaction

The PCR mixture contained 5 µg/ml of 5'-primer, 5 µg/ml of 3'-primer, 1.5 mM $MgCl_2$, 200 µM of each of the nucleotides dATP, dCTP, dGTP and dTTP (Boehringer Mannheim, Almere, the Netherlands), and 2 U AmpliTaq Gold DNA polymerase (Perkin Elmer, Nieuwerkerk a/d IJssel, the Netherlands) in 1*PCR buffer II (Perkin Elmer). Primers were synthesized by Gibco, and are listed in Table 1. PCR was performed by incubation for 12 min at 94°C, followed by 33 and 36 cycles each of 45 sec at 94°C, 45 sec at 60°C, and 2 min at 72°C, and a final incubation for 7 min at 72°C. PCR products were electrophorized on a 2 % agarose gel and visualized by ethidium bromide staining. PCR for HPRT was used as a control for the cDNA synthesis. Ladder is marker IX (72-1353 bp; Boehringer, Mannheim, Germany).

Table 1
PCR primers used in this study.

Gene	Sequence 5'primer	Sequence 3'primer	Length (bp)	Number of cycles[a]
IL-1α	TTACAGTGAAAACGAAGA	TGTTTGTCCACATCCTG	417	33
IL-1β	TTGACGGACCCCAAAAGATG	AGAAGGTGCTCATGTCCTCA	204	33
IL-2	GACACTTGTGCTCCTTGTCA	TCAATTCTGTGGCCTGCTTG	227	n.d.
IL-4	CCAGCTAGTTGTCATCCTGCT CTTCTTTCTCG	CAGTGATGTGGACTTGGACTC ATTCATGGTGC	384	n.d.
IL-6	GTTCTCTGGGAAATCGTGGA	TGTACTCCAGGTAGCTATGG	208	36
IL-10	ATGCAGGACTTTAAGGGTTAC TTG	TAGACACCTTGGTCTTGGAGC TTA	254	36
IL-12 p35	AACAGCCTCACCCTCGGC	GTTGATGGTCACGACGCG	258	36
IL-12 p40	CAGTACACCTGCCACAAAGGA	GTGTGACCTTCTCTGCAGACA	277	36
IL-18	ACAAAGAAAGCCGCCTCAAAC	GTCTGGTCTGGGGTTCACTGG	294	33
GM-CSF	TCTCATCAGTTCTATGGCCC	GGGAGTAGACAAGGTACAAC	212	33
IFN-γ	TTCCTGGGCATTGTGGTCT	TGGATTCAGAGCTGGCCTGG	431	n.d.
MIP-2	TGCATCTTGGTCTTGCAGCTCT TCCTCATGGC	TGGACCTGTGGGTTGTTGACC TCAACCTTGGC	365	33
TNF-α	GCCAGTGAACTGCGCTGTCAA TGC	GTTAGCCTTGCCTTTGTTCAGT ATG	211	33
HPRT	GTCAAGCAGTACAGCCCCAAA ATGG	TAGTGCAAATCAAAAGGGAC GCAGC	385	30

n.d. not detected
[a] minimal number of cycles needed for detection

ELISA

For cytokine determinations, ELISA reagents from R&D Systems (Oxon, UK) were used in accordance to the manufacturer`s specifications. Briefly, 96-well plates (Nunc-Immuno Plate, Roskilde, Denmark) were coated with the capture antibody in PBS and incubated overnight at room temperature (RT). The plates were blocked by adding PBS containing 1% bovine serum albumin (BSA; Sigma, Axel, the Netherlands) plus 0.05% Tween-20 (Merck, in PBS) for 2 hr at 37°C and washed (0.05% Tween-20). Standard as well as serial dilutions of culture supernatants were added to the plate. Plates were incubated at 37°C for 2 hr and washed. Biotinylated detection antibody was added and incubated for 2 hr at RT. The plates were washed, and poly horseradish peroxidase labeled streptavidin (10,000-fold dilution, Strepta-E+, Central Laboratory of the Blood transfusion service, Amsterdam, the Netherlands) was added and incubated for 20 min at room temperature. Plates were washed again and TMB solution (0.1 mg/ml TMB (Sigma) plus 0.006% H_2O_2, in 0.1 M NaAc, pH 5.5) was added. The plates were read at 450 nm, using an automated reader (Titertek Multiskan, Flow Laboratories, Lugano, Switzerland). Antibodies, standards, samples, and streptavidin were diluted in 0.5% BSA plus 0.05% Tween-20, in PBS (Van Halteren *et al,* 1997).

Lactate dehydrogenase (LDH)

Extracellular LDH was measured after 24 hr of incubation with the test agents by measuring the enzymatic activity in the media using a commercial detection kit (Merck). Background values were subtracted from the obtained LDH concentrations. Results are presented as percentage of the total LDH content. Total LDH content was determined by incubating HEL30 cells for 2 hr with 1% Triton X-100 (Corsini *et al*, 1994). All tests were performed in duplicate. Only non-toxic concentrations were chosen based on the outcome of the LDH cytotoxicity test and are shown in Table 2.

Table 2
Concentrations used in the *in vitro* analysis.

Chemical	Concentration (µM)
Benzocaine	1400, 140, 14, 1.4
DEA	1400, 140, 14, 1.4
DNCB	7, 1.4, 0.14, 0.014
PA	1400, 140, 14, 1.4

Local lymph node assay (LLNA)

Groups of mice (n=3, 4, or 6) were pretreated with 1% SDS (w/v) one hour before exposing the animals to 25 µl of test solution in vehicle or vehicle alone on both ears daily for three consecutive days. Three days following the last topical application, the auricular lymph nodes (LN) were excised. The lymph nodes were weighed and pooled for each animal and suspended in 5 ml RPMI-1640 (Gibco) supplemented with 5% heat inactivated Fetal Calf Serum (PAA), 100 U/ml penicillin and 100 µg/ml streptomycin (standard medium). Single cell suspensions were prepared under aseptic conditions by pressing the LN through a sterile 70 µm nylon cell strainer (Falcon, Franklin Lakes, USA). The cells were washed twice in standard medium (10 minutes, 311g, 4°C) and resuspended in 1 ml standard medium with 10% FCS. The cells were

counted using a Coulter Counter and cultured at a concentration of 1.10^7 cells/ml. When necessary, cell suspensions of several animals were pooled to obtain the concentration required. The cell suspensions (200 µl) were seeded in triplicate into round-bottomed 96-well microtitre plates (Greiner, Alphen a/d Rijn, the Netherlands). The cells were cultured with 10 µl of [^{3}H]TdR (Amersham, Buckinghamshire, UK; 37 kBq/ml) for 24 hr at 37°C in a humidified atmosphere of 5% CO_2 in air. The [^{3}H]TdR incorporation was determined by liquid scintillation counting in a β plate counter (1205 Betaplate™ Wallac, Turku, Finland). The [^{3}H]TdR incorporation is expressed per animal, i.e. the [^{3}H]TdR incorporation is multiplied by the cell number of the two lymph nodes and devided by the cell number in culture.

Statistical analysis

The dose-response data were analyzed by nonlinear regression analysis, using the following family of models:

model 1: $y = a$

model 2: $y = a \exp(b x)$

model 3: $y = a \exp(b x^d))$

model 4: $y = a (c - (c - 1) \exp(b x)$

model 5: $y = a (c - (c - 1) \exp(b x^d))$.

where y represents the response and x the applied concentration. In these models the parameter *a* represents the background of the particular assay. The parameter *b* reflects the 'slope' or the 'strength' of the response with increasing dose. The selection of the model to be used for a particular data set follows from a procedure of successively fitting the above models, and applying likelihood ratio tests to establish if an increase in the number of parameters leads to a significantly better fit to the data. A model with more parameters is considered better only if this leads to a significantly better fit (Slob, 1999). The estimated concentration in % required for SI=3 (EC_3) was determined as the estimated dose inducing a stimulation index of three between treated versus control. We have used a range of test concentrations to determine dose-response relationships on which the quantitative estimation of the allergenic potency was based.

The uncertainty in the estimate of the EC_3 can be assessed by a bootstrap method (Slob and Pieters, 1998), resulting in an uncertainty distribution from which any desired confidence interval can be derived. In this paper the 5% and 95% confidense limits are reported (i.e. 90%-confidence intervals).

RESULTS

Cytokine mRNA expression and production in HEL30 cells

Cytokine mRNA expression was investigated using RT-PCR. IL-1α, IL-1β, IL-6, IL-10, IL-12 p35, IL-12 p40, IL-18, GM-CSF, MIP-2, and TNF-α mRNA expression was found in the HEL30 cell line (Figure 1). No IFN-γ, IL-2, and IL-4 mRNA expression was found (data not shown). Hypoxanthine phosphoribosyl transferase (HPRT) was used as a housekeeping control for the cDNA synthesis. Cytokines that were expressed by this cell line were analyzed on the protein level by ELISA. IL-6, IL-12 p35, and IL-12 p40 production was inconsistent, while IL-10 production was completely lacking (data not shown). A summary of cytokine mRNA expression and production is shown in Table 3.

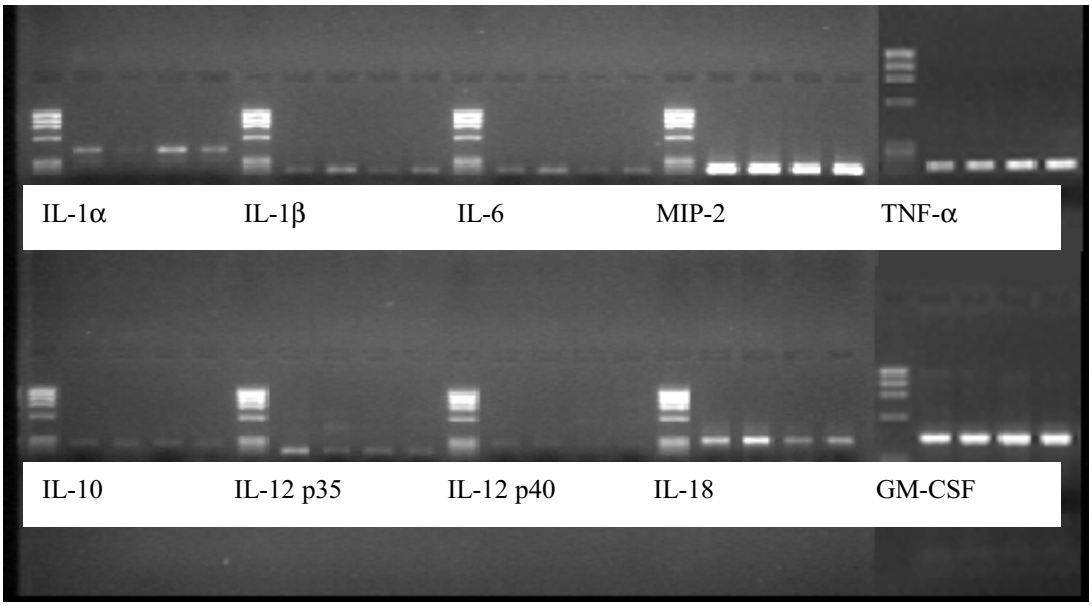

Figure 1
RT-PCR analysis of gene expression for various cytokines by the unstimulated murine keratinocyte cell line HEL30. Amplification was for 36 cycles. For each cytokine, (the lanes show from left to right) molecular weight marker, 2 and 4 µl cDNA of sample 1, and 2 and 4 µl cDNA of sample 2 are shown.

Table 3
Cytokine mRNA expression measured by RT-PCR and protein production measured by ELISA in the HEL30 cell line.

	mRNA	protein
IL-1α	yes	yes
IL-1β	yes	yes
IL-2	no	n.d.
IL-4	no	n.d.
IL-6	yes	?
IL-10	yes	?
IL-12 p35	yes	?
IL-12 p40	yes	?
IL-18	yes	yes
GM-CSF	yes	yes
IFN-γ	no	n.d.
MIP-2	yes	yes
TNF-α	yes	yes

n.d. not done
? inconsistent results

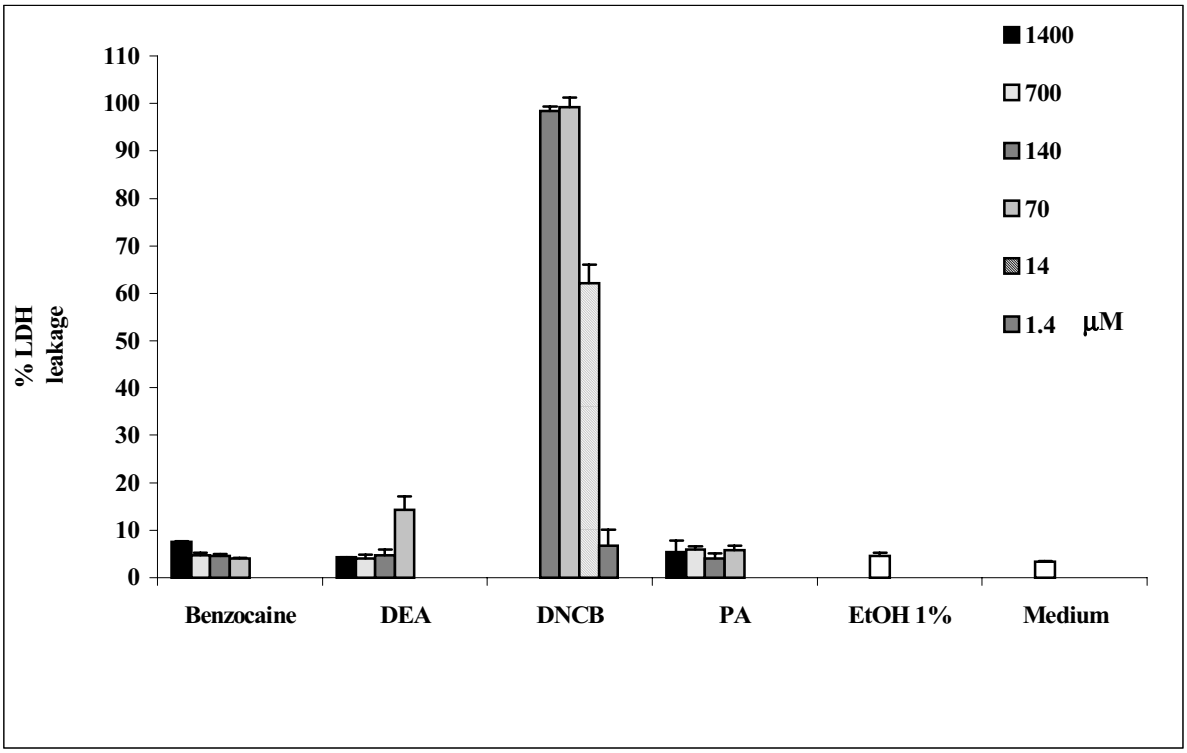

Figure 2
Leakage of lactate dehydrogenase (LDH) due to exposure to different concentrations (in µM) of various sensitizers by the HEL30 cell line. Leakage is expressed as the percentage of total LDH in the extracellular medium. Total LDH content was determined resulting from a 2 hr incubation with PBS containing 1% Triton X-100. All experiments were performed in duplicate, with similar results.

LDH leakage

Preceding the *in vitro* exposure tests, we performed a lactate dehydrogenase (LDH) leakage test to investigate the cytotoxic concentrations for the various chemicals (Figure 2). A lower intracellular cytokine content at cytotoxic concentrations is likely to result from leakage rather than secretion of IL-1α or IL-18. Leakage of the enzyme LDH after 24 hr of incubation with the various chemicals, was investigated by measuring the extracellular concentration. Leakage is expressed as the percentage of total LDH and is shown in Figure 2. No significant increase in LDH leakage compared to the 1% ethanol control was found for Benzocaine, DEA, and PA. DNCB showed 100% leakage at the two highest concentrations, while 14 µM still resulted in a leakage of approximately 60%. Only 1.4 µM DNCB showed a similar LDH leakage compared to the control. The control (1% ethanol) showed no effect compared to standard medium.

Cytokine dose-responses

Cytokines produced by the keratinocyte cell line HEL30 were analyzed by ELISA. Cytokine dose-response studies were carried out for all cytokines, except IL-2, IL-4, and IFN-γ. Both intracellular and extracellular cytokine levels were determined (data not shown). Consistent dose-response relationships were found only for intracellular IL-1α and intracellular IL-18.

The exposure effects of the various allergens on the production of intracellular IL-1α and intracellular IL-18 production is shown (Figure 3 and 4, respectively). Cytokine concentrations were measured after 24 hr of incubation to Benzocaine (panel A), DEA (panel B), DNCB (panel C), and PA (panel D). All tests were performed in triplicate. The panels show the cytokine production as a function of the dose of the various allergens on a log-scale

with the fitted regression function. The dose-response data were analyzed by nonlinear regression analysis. Clear differences in the calculated EC_3 values were observed between the different chemicals. Both IL-1α and IL-18 production showed DNCB as the allergen with the lowest EC_3 value followed in this order by PA, benzocaine, and DEA.

The EC_3 values and the associated confidence intervals for the different cytokine responses are summarized in Table 4. The chemicals are ranked according to their EC_3 values. The classification formerly obtained with the LLNA is also presented in Table 4. The ranking obtained using the in vitro studies presented here is similar to the classification derived from data formerly obtained in the LLNA.

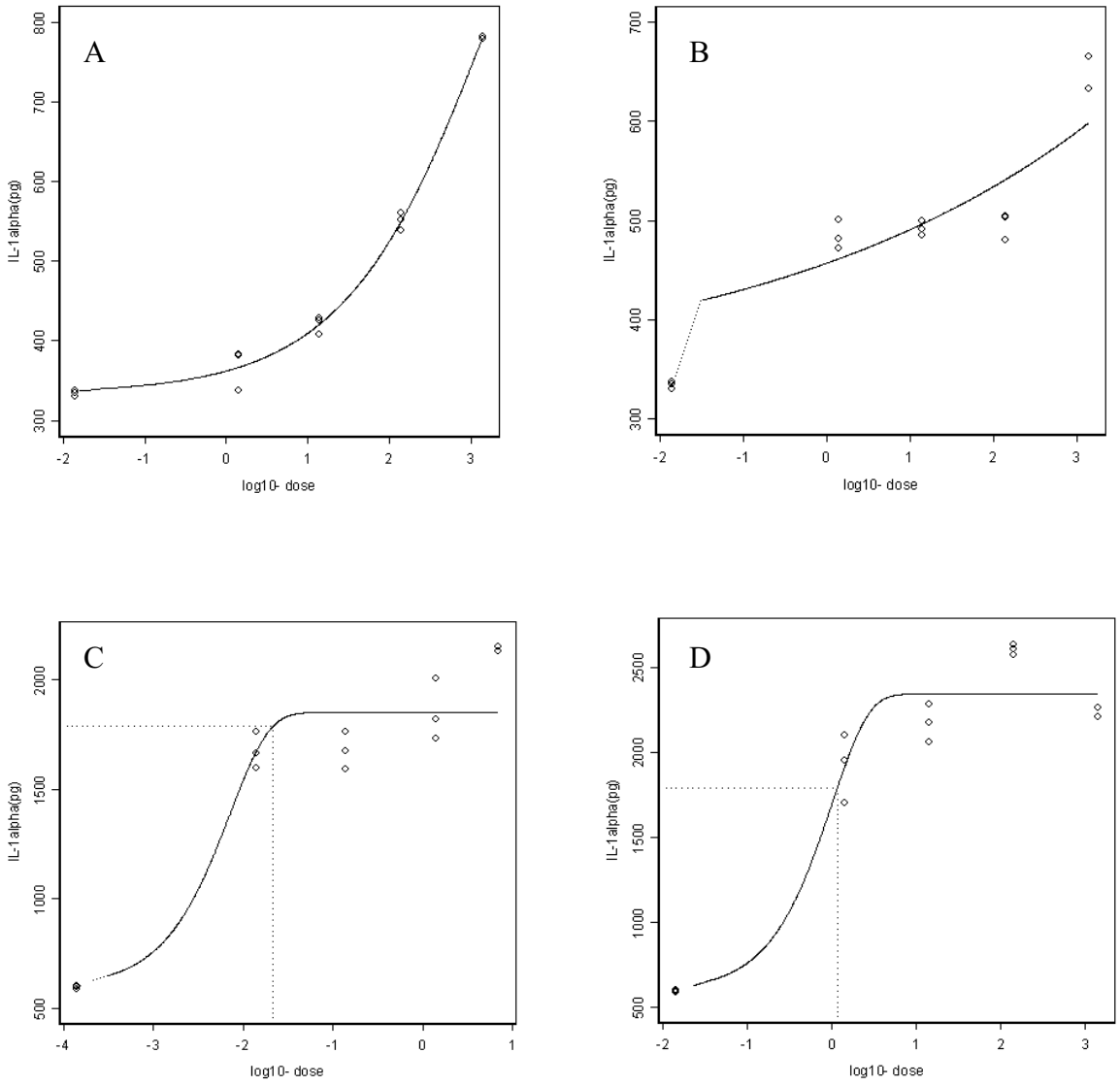

Figure 3
Intracellular IL-1α content (in pg/1.10^6 cells) in the murine keratinocyte cell line HEL30 as a function of sensitizer concentration (in μM). Using non-linear regression analysis the concentration, that induced a 3-fold increase over control, was calculated. The intracellular IL-1α concentration was measured after 24 hr of incubation to Benzocaine (A), DEA (B), DNCB (C) and PA (D). The cytokine concentrations are shown on the vertical axis on a linear scale and the concentrations of the chemicals are shown on the horizontal axis on a log-scale. Number of experiments =3.

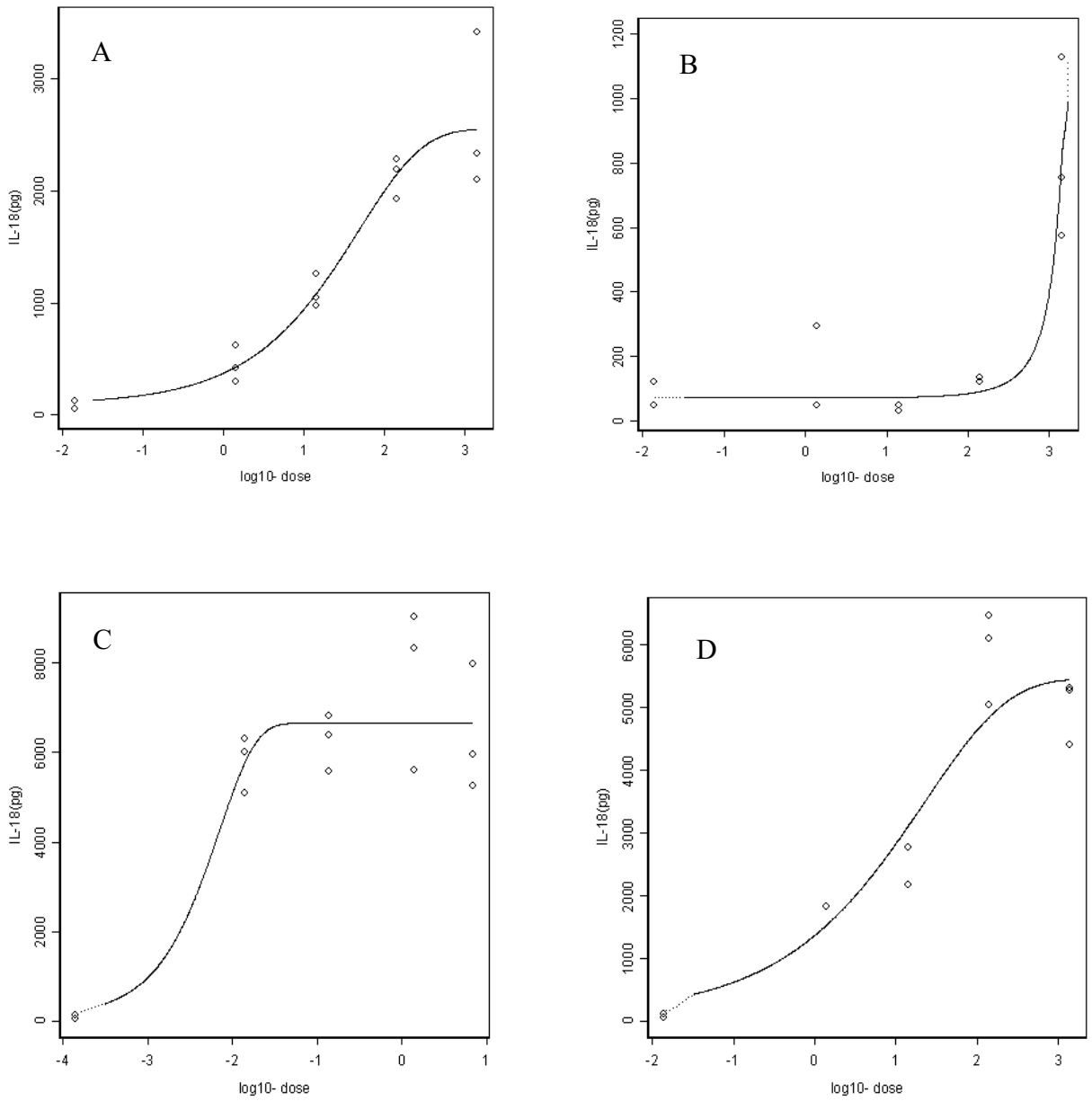

Figure 4
Intracellular IL-18 content (in pg/1.10^6 cells) in the murine keratinocyte cell line HEL30 as a function of sensitizer concentration (in μM). Using non-linear regression analysis the concentration, that induced a 3-fold increase over control was calculated. The intracellular IL-18 concentration was measured after 24 hr of incubation to Benzocaine (A), DEA (B), DNCB (C) and PA (D). The cytokine concentrations are shown on the vertical axis on a linear scale and the concentrations of the chemicals are shown on the horizontal axis on a log-scale. Number of experiments =3.

DISCUSSION

In the present study, dose-response studies were performed by exposure of the keratinocyte cell line HEL-30 to allergens of different sensitizing potency. For both intracellular IL-1α and intracellular IL-18 dose-response relationships were evaluated by nonlinear regression analysis

Table 4
Classification of low molecular weight chemicals based on cytokine responses in the HEL30 cell line and proliferative lymph node responses in the local lymph node assay (LLNA), together with the associated uncertainty distribution

Chemical	**HEL30**				**LLNA**[a]	
	IL-1α		**IL-18**			
	EC_3[b] *(μM)*	*L05-L95*[c]	EC_3 *(μM)*	*L05-L95*	EC_3 *(%)*	*L05-L95*
DNCB	$2.2*10^{-2}$	$2.4*10^{-2}$-$3.9*10^{-2}$	$3.0*10^{-5}$	NC-$3.4*10^{-4}$	0.04	0.02-0.08
PA	1.15	0.81-1.62	$8.2*10^{-3}$	$6.0*10^{-5}$-$6.3*10^{-2}$	0.36	0.23-0.56
Benzocaine	$2.4*10^{4}$	$3.4*10^{3}$-$2.9*10^{4}$	0.47	0.15-1.02	22.03	16.57-33.95
DEA	$9.9*10^{6}$	$6.0*10^{5}$-$1.5*10^{9}$	638	487-927	39.78	34.08-47.70

[a] Van Och et al., 2000.
[b] Estimated concentration required for induction of three compared to control value.
[c] 5th and 95th percentile
NC, could not be calculated

and EC_3 values were estimated based on a curve fitting method. Subsequently, these EC_3 values were used for ranking the potency of the allergens. The ranking based on these *in vitro* results corresponds well to the ranking based on the LLNA (Van Och *et al*, 2000).

A chemical that elicits a stimulation index (SI) of 3 or more in the LLNA is considered a sensitizer. Moreover, the concentration necessary to induce this SI can be employed for ranking (Basketter *et al*, 2000; Van Och *et al*, 2000). In the *in vitro* studies described in this paper, we chose a SI of three (over vehicle control) of IL-1α and IL-18 production. If a SI=2 or SI=5 were chosen, a similar ranking was obtained.

Epidermal keratinocytes constitutively synthesize IL-1α (Dinarello, 1984), and contain a large amount of preformed and biologically active IL-1α (Mizutani, 1991). Once the physical barrier of the upper epidermis is damaged, IL-1α is rapidly released so that this IL-1α reservoir represents a rapid response system for immune and inflammatory regulation in the skin, and IL-1α acts as a first line of defence against injury (Kupper, 1988). We found a consistent dose-response relationship for IL-1α. Previous findings using the HEL30 cell line showed that only contact allergens were able to increase intracellular IL-1α in a dose dependent fashion (Corsini *et al*, 1998). Besides IL-1α, we found a consistent dose-response relationship for IL-18, a cytokine that was originally discovered to induce IFN-γ production from Th1 and NK cells (Okamura *et al*, 1995; 1998). Recent studies revealed that IL-18 also induces the production of Th2 cytokines from T cells, NK cells, basophils, and mast cells (Hoshino *et al*, 1999; Yoshimoto *et al*, 1999; Nakanishi *et al*, 2001). Also IL-18 is known to be constitutively produced by keratinocytes (Stoll *et al*, 1997). IL-18 acts synergistically with IL-12 to induce a Th1 response through induction of IFN-γ by T-lymphocytes (Micallef *et al*, 1996; Robinson *et al*, 1997), and might result in induction of IL-4 and IL-13 in the skin in an IgE-independent manner, directly inducing allergic inflammatory responses (Nakanishi *et al*, 2001). Recent findings reveal that contact allergen-induced LC migration and dendritic cell accumulation in draining lymph nodes require IL-18 (Cumberbatch *et al*, 2001), important for the development of allergic responses in the skin. We found low baseline levels of intracellular IL-18, but a considerable increase as a result of allergen exposure. This is in accordance with previous findings observed in another murine keratinocyte cell line, being PAM 212, where contact allergens, but not irritants, induced expression and production of IL-18 (Stoll *et al*, 1997).

Both IL-1α and IL-18 seem to be good parameters for allergenic potency, as clear differences are seen between the various allergens (Table 4). IL-18 seems to be a more sensitive parameter compared to IL-1α. As 10 M of DEA was needed to obtain a SI=3 for IL-1α production, only a fraction of that amount (0.64 mM) was needed to induce a similar induction of IL-18 production.

The quality of the data can be translated to uncertainty margins which benefits the comparison between the sensitizers (Van Och *et al*, 2000). The uncertainty in the estimate of the EC_3 was assessed by a bootstrap method (Slob and Pieters, 1998), resulting in an uncertainty distribution from which any desired confidence interval can be derived. This bootstrap method was performed for both IL-1α and IL-18 responses. No overlap of the confidence intervals was found for the different sensitizers, which underscores the reliability of the ranking. Except DNCB and PA show for IL-18 production a small overlap of the confidence intervals.

Whereas murine keratinocytes do not express IL-1β (Ansel *et al*, 1988), we found both mRNA expression and production of the protein in the HEL30 cell line. Biologically inactive pro-IL-1β has to be cleaved into the active form by the intracellular protease IL-1β-converting enzyme (ICE, recently termed caspase-1). Although murine keratinocytes synthesize caspase-1 (Ariizumi *et al*, 1995), it is unlikely that this is constitutively active since mature IL-1β is not produced. However, while also IL-18 relies on caspase-1 (Ghayur *et al*, 1997) for processing in these cells, biologically active IL-18 is found. This contradiction is difficult to explain. Possibly, HEL30 cells are different in that respect compared to normal murine keratinocytes.

A logical next step is to perform similar *in vitro* studies with human keratinocytes and compare the differences in potency with the outcome of the present study and the ranking formerly obtained in the LLNA. Data derived from the analysis in human cells may also provide us with information about differences in sensitivity between murine and human keratinocytes. This can be helpful in the calculation or estimation of extrapolation factors, which can be used to translate *in vivo* data (LLNA) towards an actual risk estimate in man.

The ranking of the allergens based on the EC3 values derived from the IL-1α and IL-18 dose-response relationships corresponded to the *in vivo* classification data based on the LLNA. These data support the hypothesis that the irritating component of an allergen decides to a great extent the ultimate strength of the sensitizing effect. Therefore, IL-1α and IL-18 production by keratinocytes may be seen as a possible tool for the prediction of sensitizing strength of a particular chemical. Such *in vitro* testing may be useful for ranking chemicals for their sensitizing potential resulting in a reduction of animal testing.

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CHAPTER 9

CYTOKINE RESPONSES IN HUMAN KERATINOCYTES BY EXPOSURE TO LOW MOLECULAR WEIGHT CHEMICALS: PREDICTIVE VALUE AND A TOOL FOR EXTRAPOLATION

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The final step of the immunotoxicity risk assessment process is risk characterization. Unfortunately, the current lack of adequate human data is a major limitation. To rely on animal data, important for risk characterization are dose-response relationships and tools for extrapolation from experimental animals to humans. In the present study we used a test system comprised of the human epidermal keratinocyte cell line HaCaT. We exposed this cell line to the allergens ethyl-p-aminobenzoate (benzocaine), diethylamine (DEA), 2,4-dinitrochlorobenzene (DNCB), and phthalic anhydride (PA). IL-1α and IL-18 dose-response data were evaluated by non-linear regression analysis, and at a ratio of three of cytokine production of treatment versus control, the corresponding allergen concentration was calculated. For IL-18, DNCB showed the strongest potency followed in this order by PA, benzocaine, and DEA. This classification was similar to our previous findings obtained in a modified local lymph node assay (LLNA). Dose-response analysis of IL-1α ranked benzocaine more potent than PA. The ranking based on the in vitro results in the human cell line HaCaT corresponded to a large extent to the results obtained in experimental animals (LLNA). In addition, cytokine responses by the human HaCaT cell line were compared to responses of the murine epidermal keratinocyte cell line HEL30 in order to obtain information on differences in sensitivity to sensitizers between humans and mice. The HaCaT cell line showed to be less sensitive compared to its murine counterpart. This in vitro approach not only provides significant knowledge on the predictive value of animal tests, it can also provide us with a tool for improved extrapolation from animals to the human situation.

INTRODUCTION

The process of risk assessment from chemical exposures can be divided into four different steps: (1) hazard identification; (2) hazard characterization; (3) exposure assessment; and (4) risk characterization. Traditionally, in vitro studies have most frequently been applied for hazard identification purposes. However, over the last 20 years there has been an increased recognition that *in vitro* studies can also be of use for risk characterization (Holme and Dybing, 2002). Human cell and tissue models are being used increasingly for prediction of drug-specific organ toxicities, to predict human metabolism, and to identify potential drug-drug interactions. Establishment of the dose-response relationships for critical effects and extrapolation from experimental animals to humans are two elements important in hazard characterization (European Commission, 2000).

The local lymph node assay (LLNA) is now commonly used for the identification of the sensitising activity of chemicals and the assay has been validated to the guinea pig maximization test (Basketter and Scholes, 1992; Basketter *et al.*, 1993; Kimber *et al.*, 1995). Test chemicals are applied to the dorsum of the ear and lymphocyte activation is determined by measuring cell proliferation in the draining auricular lymph nodes, which can be done either by *in vivo* or *ex vivo* tritium-thymidine labeling of the cells (Kimber and Weisenberger, 1989; Kimber *et al.,* 1995; Van Och *et al.,* 2000). Analysis of 134 chemicals tested in the local lymph node assay (LLNA), guinea pig maximisation test (GPMT) and/or with clear clinical evidence for human skin sensitisation potential, revealed that an EC_3 (effective concentration causing a stimulation index of 3 compared to vehicle control) in the LLNA is an acceptable threshold value for hazard identification (Basketter *et al.*, 1999a). In the LLNA quantitative data are obtained on the induction phase of the immune response. So, based on the EC_3 value an accurate assessment of sensitising potency is possible (Basketter *et al.*, 1999b; Van Och *et al.*, 2000).

Classification of the allergenic potency of a chemical in humans is problematic, since it depends on a number of endogenous and exogenous factors (Jerschow *et al.*, 2001) which results in a current lack of adequate human data. To overcome this difficulty, an alternative type of evaluation of the risk of adverse effects due to exposure to (immunotoxic) chemicals may be used; the so-called parallellogram approach (Blaauboer *et al*., 1990; Van Loveren *et al*., 1997). It is possible to compare the results of *in vitro* and *in vivo* animal studies using the same or similar endpoints of immunotoxicity, as it is possible to compare the results of *in vitro* studies in animals and in man. Based on the combined comparison of these results, an improved extrapolation from animals to the human situation is thus achievable.

In the present study, a spontaneously immortalized human keratinocyte cell line, HaCaT, was used as an in vitro model to predict the sensitizing potency of allergens. It has been hypothesized that the irritant capacity of allergens presents an additional risk factor (Cumberbatch *et al*., 1993; Grabbe *et al*., 1996). This suggests that evaluating irritant potency of allergenic chemicals may be helpful to establish their allergenic potency. Epidermal keratinocytes are known to produce and excrete a wide range of cytokines (Haas *et al*, 1992). Therefore, it can be suggested that the potency of chemicals to induce cutaneous sensitization can be assessed as a function of keratinocyte cytokine expression. The anatomical location of keratinocytes and their significant role in the development of allergic contact dermatitis, justify the use of keratinocyte-derived cytokine production as read-out to evaluate sensitizing potency.

Cells were exposed to benzocaine, DEA, DNCB and PA, known allergens with different sensitizing potency (Van Och *et al*, 2000). The cytokine dose-response data were evaluated by non-linear regression analysis and at a stimulatory index (SI) of three the

corresponding estimated concentration was calculated (EC_3). We used these EC_3 values to rank these chemicals and to compare the outcome of the present study with those obtained from the local lymph node assay (LLNA). These data will tell us whether the ranking based on the cytokine responses in the human keratinocyte cell line is in accordance with the ranking derived in the mouse (*in vivo*). In addition, the EC_3 values obtained in the human HaCaT cell line were compared to EC_3 values previously obtained in the murine HEL30 cell line (Van Och *et al*., submitted). This comparison may provide information on the differences in sensitivity between the two species mouse and man. Based on the combined comparison of these results, an improved extrapolation from animals to humans may be achieved.

MATERIALS AND METHODS

Chemicals

Benzocaine (ethyl-p-aminobenzoate; 99% purity; Sigma-Aldrich Chemie B.V., Zwijndrecht, the Netherlands), DEA (diethylamine; 99.5%; free base solution; Sigma-Aldrich), DNCB (2,4-dinitrochlorobenzene; 98%; Sigma-Aldrich), and PA (phthalic anhydride; 99%; Sigma-Aldrich) were used. The chemicals were dissolved in ethanol.

Materials

The human keratinocyte cell line HaCaT and the murine keratinocyte cell line HEL30 were kindly provided by Prof. N. Fusenig (German Cancer Institute, Heidelberg, Germany).

Cell culture and exposure

Both HaCaT and HEL30 cells were cultured in Dulbecco`s modified Eagle`s medium (Gibco, Life Technologies, Breda, the Netherlands) supplemented with 10% fetal calf serum (PAA, Linz, Austria), penicillin-streptomycin (100 U/ml-0.1 mg/ml), and L-glutamine (2 mM). Cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 in air. The medium was changed twice a week and cultures were split by treating the cells with 0.25% trypsin (in PBS) for 5 min at 37°C before reaching confluency.

Cells were seeded in 12-well plates (Costar, Cambridge, MA) at a density of $1.5x10^5$/ml. After 24 hr the cells were washed once with PBS and exposed to several concentrations of various sensitizers in culture medium (Table 1). These cultures contained 1% ethanol. After 24 hr of incubation, supernatants were obtained and used for the determination of extracellular cytokine levels. The cells were then washed once with PBS and lysed with 0.5 ml 0.5% Triton X-100 (Merck, Amsterdam, the Netherlands) for 1 hr at 37°C. The cell lysate was used for determination of intracellular cytokine levels. In parallel cultures the cells were counted using a Coulter Counter (Z2, Coulter Electronics, Mijdrecht, the Netherlands). All tests were performed in triplicate. Samples were stored at -80°C until the assay was performed.

Table 1
Concentrations used in the *in vitro* analysis.

Chemical	Concentration (μM)
Benzocaine	14000, 1400, 140, 14, 1.4
DEA	14000, 1400, 140, 14, 1.4
DNCB	7, 1.4, 0.14, 0.014
PA	1400, 140, 14, 1.4

Cytokine ELISAs

For cytokine determinations, ELISA reagents from R&D Systems (Oxon, UK) were used in accordance to the manufacturer`s specifications. Briefly, 96-well plates (Nunc-Immuno Plate, Roskilde, Denmark) were coated with the capture antibody in PBS and incubated overnight at room temperature (RT). The plates were blocked by adding PBS containing 1% bovine serum albumin (BSA; Sigma, Axel, the Netherlands) plus 0.05% Tween-20 (Merck, in PBS) for 2 hr at 37°C and washed (0.05% Tween-20). Standard as well as serial dilutions of culture supernatants were added to the plate. Plates were incubated at 37°C for 2 hr and washed. Biotinylated detection antibody was added and incubated for 2 hr at RT. The plates were washed, and poly horseradish peroxidase labeled streptavidin (10,000-fold dilution, Strepta-E+, Central Laboratory of the Blood transfusion service, Amsterdam, the Netherlands) was added and incubated for 20 min at room temperature. Plates were washed again and TMB solution (0.1 mg/ml TMB (Sigma) plus 0.006% H_2O_2, in 0.1 M NaAc, pH 5.5) was added. The plates were read at 450 nm, using an automated reader (Titertek Multiskan, Flow Laboratories, Lugano, Switzerland). Antibodies, standards, samples, and streptavidin were diluted in 0.5% BSA plus 0.05% Tween-20, in PBS (Van Halteren *et al.,* 1997).

Local lymph node assay (LLNA)

Groups of mice (n=3, 4, or 6) were pretreated with 1% SDS (w/v) one hour before exposing the animals to 25 µl of test solution in vehicle or vehicle alone on both ears daily for three consecutive days. Three days following the last topical application, the auricular lymph nodes (LN) were excised. The LN were weighed and pooled for each animal and suspended in 5 ml RPMI-1640 (Gibco) supplemented with 5% heat inactivated Fetal Calf Serum (PAA), 100 U/ml penicillin and 100 µg/ml streptomycin (standard medium). Single cell suspensions were prepared under aseptic conditions by pressing the LN through a sterile 70 µm nylon cell strainer (Falcon, Franklin Lakes, USA). The cells were washed twice in standard medium (10 minutes, 311g, 4°C) and resuspended in 1 ml standard medium with 10% FCS. The cells were counted using a Coulter Counter and cultured at a concentration of 1.10^7 cells/ml. When necessary, cell suspensions of several animals were pooled to obtain the concentration required. The cell suspensions (200 µl) were seeded in triplicate into round-bottomed 96-well microtitre plates (Greiner, Alphen a/d Rijn, the Netherlands). The cells were cultured with 10 µl of [^{3}H]TdR (Amersham, Buckinghamshire, UK; 37 kBq/ml) for 24 hr at 37°C in a humidified atmosphere of 5% CO_2 in air. The [^{3}H]TdR incorporation was determined by liquid scintillation counting in a β plate counter (1205 Betaplate^{TM} Wallac, Turku, Finland). The [^{3}H]TdR incorporation is expressed per animal, i.e. the [^{3}H]TdR incorporation is multiplied by the cell number of the two LN and devided by the cell number in culture.

Statistical analysis

The dose-response data were analyzed by nonlinear regression analysis, using the following family of models:

model 1: $y = a$

model 2: $y = a \exp(b x)$

model 3: $y = a \exp(b x^d))$

model 4: $y = a (c - (c - 1) \exp(b x)$

model 5: $y = a (c - (c - 1) \exp(b x^d))$.

where y represents the response and x the applied concentration. In these models the parameter *a* represents the background of the particular assay. In all models the parameter *a* is constrained to being larger than zero, since it denotes the value of the response at dose zero. The parameter *b* reflects the 'slope' or the 'strength' of the response with increasing dose.

Parameter *c* determines whether the function increases or decreases, by being larger or smaller than unity, respectively. To render model 3 describing a decreasing response *d* has to be changed into *-d*. The parameter *d* is constrained to values larger than (or equal to) one, to prevent the slope of the function at dose zero from being infinite, this being biologically unreal. The selection of the model to be used for a particular data set follows from a procedure of successively fitting the above models, and applying likelihood ratio tests to establish if an increase in the number of parameters leads to a significantly better fit to the data. A model with more parameters is considered better only if this leads to a significantly better fit (Slob, 1999). The selected model is used to derive the estimated concentration (EC) associated with a stimulation index or cytokine production. Full details on these statistics are given in Slob (2002).

The uncertainty in the estimate of the EC_3 can be assessed by a bootstrap method (Slob and Pieters, 1998), resulting in an uncertainty distribution from which any desired confidence interval can be derived. In this paper the 5% and 95% confidense limits are reported (i.e. 90%-confidence intervals).

RESULTS

Preceding the *in vitro* exposure tests, for the various chemicals the cytotoxic concentrations were determined by measuring lactate dehydrogenase (LDH) leakage (data not shown). For the various chemicals, the highest non-toxic concentration was chosen as the highest concentration of exposure. Remarkably, the highest non-toxic concentration of DNCB was 200- to 2,000-fold lower compared to the other chemicals tested. In preliminary studies, we have tested a range of cytokines in dose-response studies. (Only) intracellular IL-1α and intracellular IL-18 showed clear dose-response relationships (data not shown).

Potency evaluation

Dose-response studies measuring intracellular cytokines were carried out for IL-1α and IL-18 and are shown in Figure 1 and 2, respectively. Cytokine concentrations were measured after 24 hr of incubation to Benzocaine (panel A), DEA (panel B), DNCB (panel C), and PA (panel D). All tests were performed in triplicate. The panels show the cytokine production as a function of the dose of the various allergens on a log-scale with the fitted regression function.

Both cytokines showed consistent dose-response relationships for DNCB and PA. The chemicals benzocaine and DEA showed a significant increase in cytokine production only for the highest concentration used (14 mM). The dose-response data were analyzed by nonlinear regression analysis. Differences in the calculated EC_3 values were observed between the different chemicals. For IL-18, DNCB showed the the lowest EC_3 value followed in this order by PA, benzocaine, and DEA. For IL-1α, the same order was seen, except for benzocaine that showed a lower EC_3 value compared to PA.

The EC_3 values and the associated confidence intervals for the different cytokine responses are summarized in Table 2. The chemicals are ranked according to their EC_3 values. The classification formerly obtained with the LLNA is also presented in Table 2. The ranking obtained using the *in vitro* studies presented here is similar to the classification derived from data formerly obtained in the LLNA.

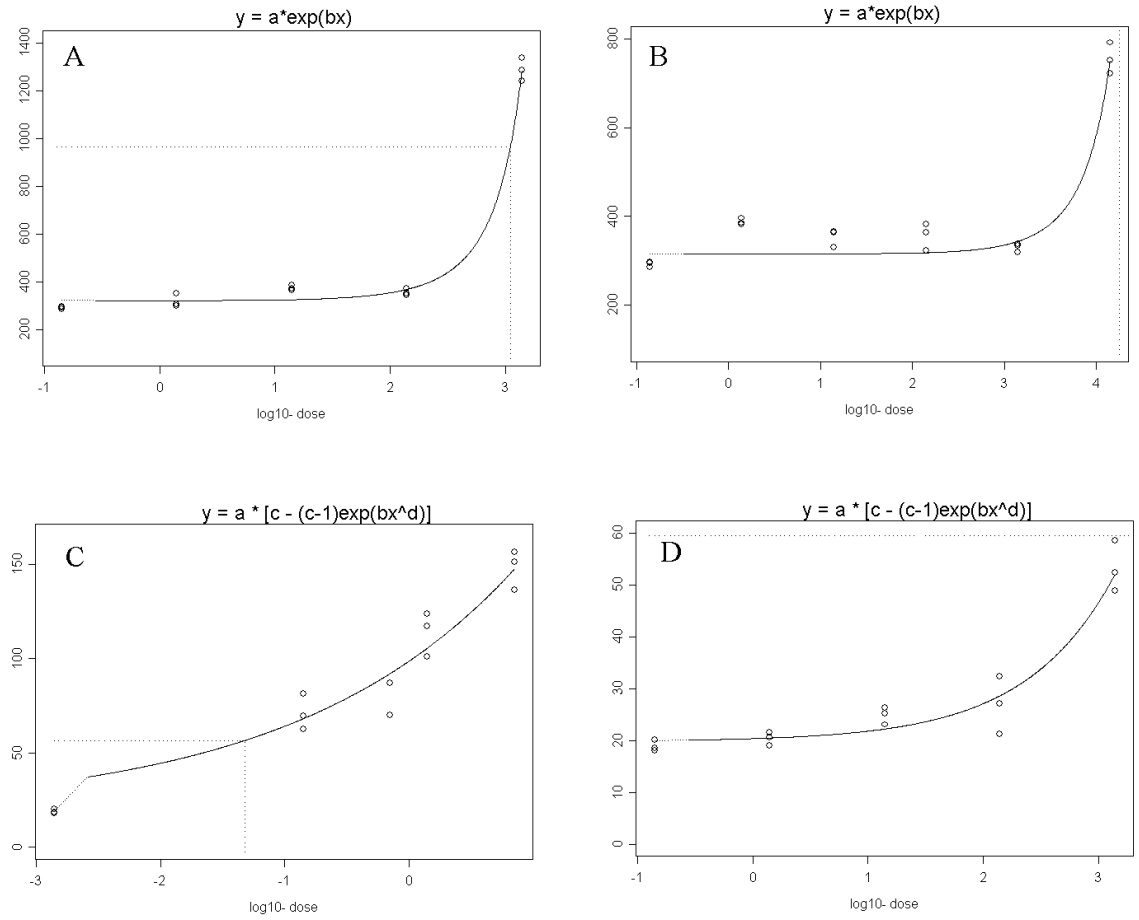

Figure 1
Intracellular IL-1α content (in pg/1.10^6 cells) in the human keratinocyte cell line HaCaT as a function of sensitizer concentration (in μM). Using non-linear regression analysis the concentration, that induced a 3-fold increase over control, was calculated. The intracellular IL-1α concentration was measured after 24 hr of incubation to Benzocaine (A), DEA (B), DNCB (C) and PA (D). The cytokine concentrations are shown on the vertical axis on a linear scale and the concentrations of the chemicals are shown on the horizontal axis on a log-scale. Number of experiments =3.

Species comparison *in vitro*

The EC_3 values measured in the human keratinocyte HaCaT cell line after exposure to Benzocaine, DEA, DNCB, and PA were compared to EC_3 values previously obtained in the murine keratinocyte HEL30 cell line. These observations are summerized for IL-1α and IL-18 in Table 3 and 4, respectively. Based on the outcome of the LDH test, the highest non-toxic concentration chosen for benzocaine and DEA in the human cell line, were one order of magnitude higher (14 mM) compared to the highest non-toxic concentration chosen in the murine cell line (1.4 mM). In contrast to HEL30, HaCaT showed clear differences in the calculated EC_3 values (without overlap of the confidence intervals) between the different chemicals. In the HaCaT cell line, for IL-1α, an overlap of the 90%-confidence interval was seen for PA and benzocaine, whereas for IL-18 an overlap was seen for benzocaine and DEA. In the HaCaT cell line a smaller range of the cytokine responses was found between the lowest

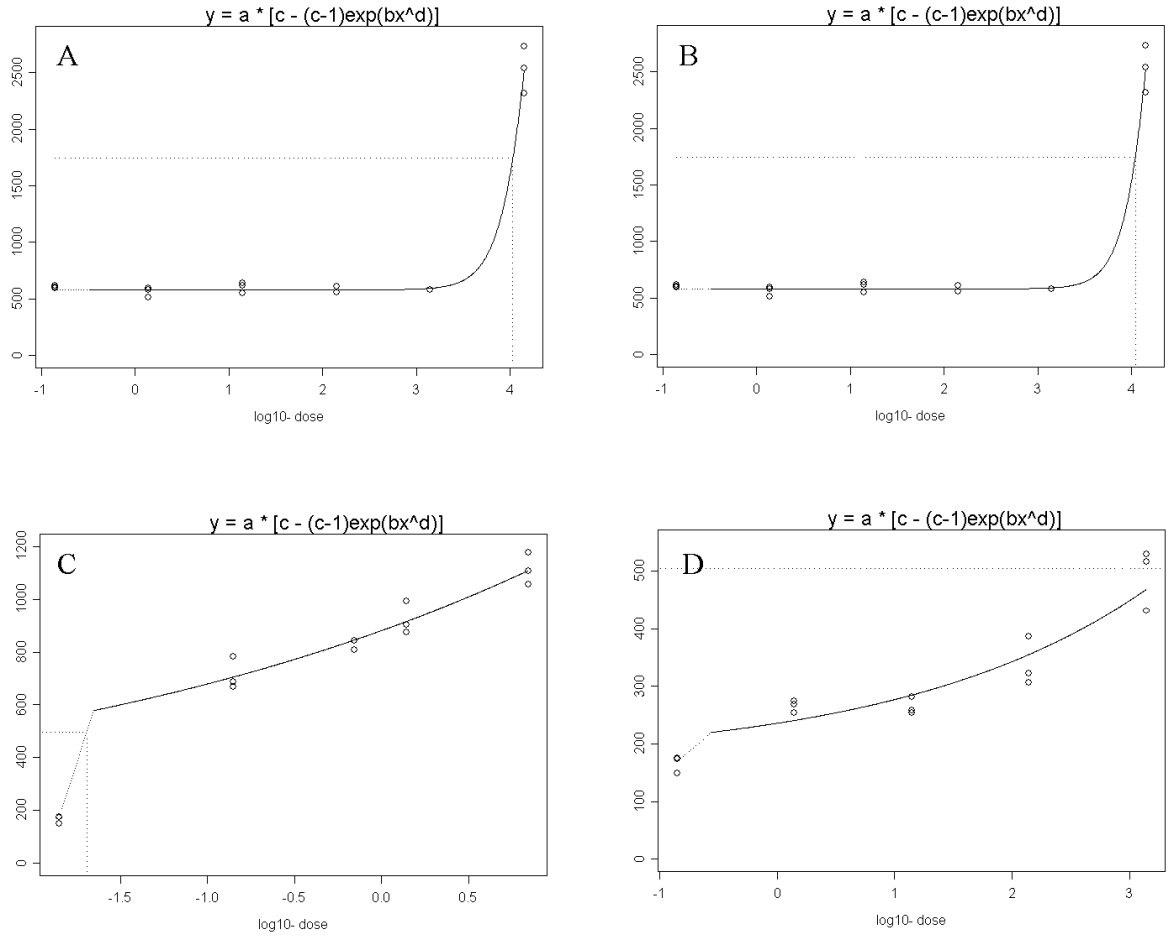

Figure 2
Intracellular IL-18 content (in pg/1.10^6 cells) in the human keratinocyte cell line HaCaT as a function of sensitizer concentration (in μM). Using non-linear regression analysis the concentration, that induced a 3-fold increase over control was calculated. The intracellular IL-18 concentration was measured after 24 hr of incubation to Benzocaine (A), DEA (B), DNCB (C) and PA (D). The cytokine concentrations are shown on the vertical axis on a linear scale and the concentrations of the chemicals are shown on the horizontal axis on a log-scale. Number of experiments =3.

and the highest concentration tested, and also between the strongest and the weakest allergen tested.

DISCUSSION

In the present study, we investigated if the ranking found *in vitro* using production of cytokines by human keratinocytes corresponded to the findings previously obtained in experimental animals (LLNA). Dose-response studies were performed by exposure of the human keratinocyte cell line HaCaT to allergens of different sensitizing potency. Intracellular IL-1α and IL-18 dose-response relationships were evaluated by nonlinear regression analysis and EC_3 values were estimated based on a curve fitting method. Subsequently, these EC_3 values were used for ranking the potency of the allergens. Uncertainty in the estimate of the EC_3 was assessed by a bootstrap method (Slob and Pieters, 1998), from which any desired

confidence interval can be derived. For both IL-1α and IL-18, DNCB showed the strongest potency followed in this order by PA, benzocaine, and DEA. Dose-response analysis of IL-1α ranked benzocaine more potent than PA. However, an overlap of the confidence intervals was seen between benzocaine and PA, suggesting no clear difference in potency between these chemicals. This may be the result of the use of two different models (Figure 1) for the analysis of the dose-response data of these two chemicals. So in general, these classifications were similar to our previous findings obtained in the LLNA (Van Och *et al*., 2000).

Table 3
Classification of low molecular weight chemicals based on IL-1α responses in the human HaCaT and the murine HEL30 cell line, together with the associated uncertainty distribution.

Chemical	*HaCaT*		*HEL30*[c]	
	EC_3[a] *(μM)*	*L05-L95*[b]	EC_3 *(μM)*	*L05-L95*
DNCB	$4.8*10^{-2}$	$1.6*10^{-2}$-$1.3*10^{-1}$	$2.2*10^{-2}$	$2.4*10^{-2}$-$3.9*10^{-2}$
PA	$2.0*10^{3}$	$1.4*10^{3}$-$3.3*10^{3}$	1.15	0.81-1.62
Benzocaine	$1.1*10^{3}$	$1.0*10^{3}$-$1.2*10^{3}$	$2.4*10^{4}$	$3.4*10^{3}$-$2.9*10^{4}$
DEA	$1.8*10^{4}$	$1.5*10^{4}$-$2.1*10^{4}$	$9.9*10^{6}$	$6.0*10^{5}$-$1.5*10^{9}$

[a] Estimated concentration required for induction of three compared to control value
[b] 5th and 95th percentile
[c] Van Och et al., submitted

Table 4
Classification of low molecular weight chemicals based on IL-18 responses in the human HaCaT and the murine HEL30 cell line, together with the associated uncertainty distribution.

Chemical	*HaCaT*		*HEL30*[c]	
	EC_3[a] *(μM)*	*L05-L95*[b]	EC_3 *(μM)*	*L05-L95*
DNCB	$4.9*10^{-3}$	$1.2*10^{-3}$-$2.6*10^{-2}$	$3.0*10^{-5}$	NC-$3.4*10^{-4}$
PA	$2.4*10^{3}$	$1.0*10^{3}$-$7.1*10^{3}$	$8.2*10^{-3}$	$6.0*10^{-5}$-$6.3*10^{-2}$
Benzocaine	$10.6*10^{3}$	$10.6*10^{3}$-$11.1*10^{3}$	0.47	0.15-1.02
DEA	$10.9*10^{3}$	$10.8*10^{3}$-$11.3*10^{3}$	638	487-927

[a] Estimated concentration required for induction of three compared to control value
[b] 5th and 95th percentile
[c] Van Och et al., submitted
NC, could not be calculated

Although it is helpful to have available *in vitro* methods to assess skin sensitization potential, it would be even more valuable to have available methods that can be used to extrapolate data from animal studies to humans (Gerberick and Robinson, 2000). Data derived from the analysis in human cells may provide us with information about differences in sensitivity between murine and human keratinocytes and may therefore be helpful in the calculation or estimation of extrapolation factors. For HaCaT, the highest non-toxic concentrations were found to be one order of magnitude higher for benzocaine and DEA, while for DNCB and PA similar concentrations were used for both HaCaT and HEL30 cells. In addition, a smaller range of cytokine responses was found in the HaCaT cell line, both between the lowest and the highest concentration tested and between the strongest and the weakest allergen tested as compared to similar responses in murine HEL30 cells. For IL-18, for each chemical only a fraction of the concentration is needed for a three-fold induction over vehicle control in the HEL30 cell line compared to the HaCaT cell line. For IL-1α, this was only seen for DNCB and PA. These observations indicate that the human HaCaT cell line showed less

sensitive responses compared to the murine HEL30 cell line. This may suggest that mice are more sensitive than humans. It should however be noted that the difference in overall sensitivity may be a function of intrinsic capacity of the cell lines, rather than indicate species differences. The use of primary keratinocytes may overcome this problem.

In conclusion, the ranking observed in the human *in vitro* model based on the EC_3 values derived from the IL-1α and IL-18 dose-response relationships corresponded in the human keratinocyte cell line HaCaT with the ranking previously obtained in the LLNA and with the ranking obtained from murine HEL30 cells. These data not only support the hypothesis that the irritant component of an allergen decides to a great extent the ultimate strength of the sensitizing effect, it also underscores the predictive value of the LLNA. In addition, the *in vitro* approach can also provide us with missing information on differences in sensitivity between murine and human keratinocytes. Our investigations showed less sensitive responses in the human cell line compared to the murine cell line. This may be helpful in the calculation or estimation of extrapolation factors, which can be used to translate *in vivo* data (LLNA) towards an actual risk estimate in man. However, tumor cell lines were used in the present approach, and further validation with primary keratinocytes is necessary.

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CHAPTER 10

SUMMARY AND GENERAL DISCUSSION

The skin not only functions as a physical-mechanical barrier between an individual and his/her environment, it also acts as an immunologically active organ. Allergic skin diseases can be quite incapacitating and form a serious occupational and consumer health problem. One of the most important allergic diseases caused by chemicals, contact hypersensitivity (CHS) or skin sensitization, forms a serious problem for individuals experiencing such a reaction. The prevalence of CHS tends to grow proportionally to the increasing exposure to an expanding variety of chemicals. Predictive animal tests to identify sensitizing properties of chemicals are therefore carried out at a large scale. For a better risk assessment, a more quantitative assessment of the sensitizing potency of chemicals is needed. These two current developments result in an increasing number of animals used. The aim of this thesis was to evaluate the risk assessment process for sensitizing potency of contact allergens in a more reliable manner, and to investigate the possibility of developing alternative approaches to *in vivo* testing, which may ultimately lead to a refinement, reduction, or replacement of animal testing.

The animal tests most commonly used to identify skin sensitizing capacity are the guinea pig maximization test (GPMT; Magnusson and Kligman, 1969, 1970) and the local lymph node assay (LLNA; Kimber *et al.*, 1986; Kimber and Weisenberger, 1989). The murine LLNA has been recently accepted as a stand-alone test (OECD 1992; OECD 2000). It investigates sensitization potential by measuring cell proliferation in the lymph node (LN) draining the site of application, being the ear. In **chapter 2** the sensitizing potential of ten model allergens was evaluated using a dose-response analysis on data obtained using the LLNA. Usually, the stimulation index (SI) of three is used as a cut-off in the LLNA, whereas our method evaluated all data points (animals) that contribute to the dose-response curve to determine the EC_3 (effective concentration inducing a 3-fold increase in proliferation of lymph node cells). In addition, information on the reliability of the data is obtained using a bootstrap approach (Slob and Pieters, 1998). This provides uncertainty margins which benefit the comparison between the sensitizers. The approach performed in this study is a way to accurately (with more knowledge of confidence) assess the potency of sensitizing chemicals and enables the estimation of the lowest concentration needed for sensitization. The latter approach was employed to test 15 different chemicals used in latex medical glove production (**chapter 4**). These chemicals are known to induce contact hypersensitivity reaction in man (Knudsen *et al.*, 2000). The sensitizing potency was quantified, resulting in a ranking of this potency. Now, based on the outcome of the study, chemicals preferable with lower sensitizing activity may be selected over more potent sensitizing agents for the production of natural rubber latex products.

In the standard GPMT a single concentration is used for intracutaneous and topical induction and the assay provides a qualitative assessment of allergenicity. This experimental design is not well suited for the establishment of threshold values. Therefore, we performed a quantitative evaluation of the sensitizing potency of three model allergens of different senstizing potency in the GPMT, based on multiple concentrations (**chapter 3**). Although dose-response studies with the GPMT have been described by others (Nakamura *et al.*, 1994;

Andersen *et al*., 1995), we took into account not only the percentage of reacting animals, but also the severity of the skin response. A major drawback of the GPMT, compared to the LLNA, is that skin scores are provided on an ordinal scale. Dose-response modelling of the ordinal data from the GPMT was not as successful as the dose-response modelling of the LLNA results which are continuous in nature, and we decided to apply the simple method of plotting the sum of the scores over all the animals in an experimental group against the induction dose. This resulted in a different ranking of the chemicals in the GPMT compared to the LLNA (**chapter 2**). Although dose-response modelling of ordinal data is by itself possible, the results of such an analysis are less easily interpreted. For that reason it may be hypothesized that the ranking of sensitizing chemicals is better when based on a LLNA, providing a more quantitative result on which regression analysis can be performed. Yet, the GPMT appears to reveal sensitization at lower concentrations, and may therefore be more sensitive. Besides, the GPMT measures responses in a challenge phase, which is a better reflection of the real situation when compared to the LLNA, which only measures the sensitization phase.

Previous studies have indicated that the frequency of antigen exposure is an additional risk factor in determining the outcome of the immune response (Mueller *et al*., 1989; Kitagaki *et al*., 1995). Compared to the exposure duration of the LLNA (3 days), real life exposure often lasts for months or years. Therefore, knowledge of the influence of exposure duration is necessary for an effective assessment of risk. We therefore investigated whether prolonged exposure (2 months) to sensitizers at concentrations that do not induce an SI ≥ 3 in the LLNA, was able to surpass this threshold (**chapter 5**). Prolonged exposure to concentrations of allergens below an SI of three in the LLNA, did not result in a response in the draining LN and thus were not able to surpass this threshold. This finding indicates that for classification of sensitizers the shorter exposure period in the LLNA is sufficient, and longer periods of exposure have no bearing on this classification.

The LLNA has been developed further to discriminate skin sensitizers from respiratory sensitizers (Dearman *et al*., 1992; 1996; Vandebriel *et al*., 2000). In this test, contact sensitizers have been suggested to selectively induce Th1 immune responses, such as IFN-γ production, whereas Th2 responses, such as IL-4 production, were seen after exposure to respiratory allergens (Dearman *et al*., 1995). This approach compares cytokine profiles of contact allergens and respiratory allergens based on single concentrations. Comparisons should be made based on similar SI's. Furthermore, these features may be dependent on the dose of the particular allergen. Therefore, we investigated the distinction between contact sensitizers and respiratory allergens by establishing dose-dependent cytokine profiles (**chapter 6**). Our observations indeed showed that contact allergens preferentially induce IFN-γ production, and respiratory allergens preferentally induce IL-4 and IL-10. Nevertheless, our findings also indicated that allergens of a mixed nature exist, the nature depending on the concentration used for sensitization. This finding underscores the notion to study a range of concentrations instead of only a single concentration. The use of a single short assay for both proliferation (for potency evaluation of sensitizers) and cytokine production (for discrimination between contact and respiratory sensitizers) would therefore seem a better approach and has the added advantage that it is in line with animal welfare policies.

Another approach for the identification of potential sensitizing capacity, that would not rely on *in vivo* animal (or human) testing, is directed towards the construction of alternative experimental models. Low molecular weight allergens need certain characteristics that convey them with the potential to induce allergic sensitization. Parameters that are of particular importance are protein reactivity (ability to conjugate with proteins) and lipophilicity (Basketter and Roberts, 1990). The correlation between the protein reactivity of chemicals and their skin-sensitizing potential has been well established (Dupuis and Benezra, 1982). We

sought to develop a model to incorporate a variety of molecular features, being determinants important for permeability through the skin and reactivity in the skin, to determine the allergenic potency of chemicals (**chapter 7**). Multiple regression analysis of the EC_3 with the different properties resulted in different equations from which predicted sensitising values (PSVs) could be calculated. Potency evaluation resulted in a classification of the different chemicals, in which the weak allergens could be distinguished from the strong allergens.

KC are pivotal during skin sensitization since they produce and secrete a number of proinflammatory cytokines, chemokines, and growth factors (Matsue *et al.*, 1992), and there is evidence that they play a role in immune-mediated skin diseases (Schwarz and Luger, 1992). These characteristics together with their relative ease of culture, make KC an appealing target for predictive contact sensitization testing. We investigated the relative potency of four known allergens *in vitro* based on cytokine dose-response relationships using the murine keratinocyte cell line HEL30 (**chapter 8**) and the human keratinocyte cell line HaCaT (**chapter 9**). For both intracellular IL-1α and intracellular IL-18 dose-response relationships were evaluated by nonlinear regression analysis and EC_3 values were estimated based on a curve fitting method. Subsequently, these EC_3 values were used for ranking the potency of the allergens. The ranking based on the *in vitro* results in the mouse (HEL30) corresponded to the *in vivo* classification data based on the LLNA (**chapter 2**). Subsequently, the ranking based on the *in vitro* results in the human cell line HaCaT corresponded to a large extent to the *in vitro* results found in the murine cell line HEL30.

The latter *in silico* (**chapter 7**) and *in vitro* approaches (**chapters 8 and 9**) described in the preceding alineas may be seen as possible tools for the prediction of sensitizing potency of a particular chemical, and may ultimately lead to a reduction in the use of experimental animals. Nevertheless, further validation with additional sensitizers, irritants and negative compounds is necessary. In general, major limitations and technical complexities of *in vitro* approaches are the lack of biotransformation in cultured cells, allergen toxicities, the possible non-specific immunological effects of tested chemicals, and the lack of external influences (such as other cell types or neuro-endocrine interactions). On the other hand, single-cell *in vitro* assays provide us with several advantages compared to *in vivo* assays: it saves animals, it is less expensive and time consuming, and a wider range of concentrations can be used.

With the approach described in this thesis, we are now able to assess the potency of sensitizing chemicals in the LLNA in a more accurate way. This results in a better estimation of the minimal number of test animals needed, which inevitably leads to a decrease in animal use. Another important issue is that this predictive method also provides us with information about the threshold of an allergen. The 90% confidence limits take into account the quality of the particular dataset where the lower 5% confidence limit predicts that below that particular dose there is only a 5% chance for a positive response (sensitization) and may therefore be used to define the threshold dose. Data on thresholds in humans cannot be derived directly from animal tests. Therefore, we developed an *in vitro* assay where cytokine responses derived from both murine as human keratinocytes were used as read-out for sensitizing strength. The *in vitro* approach provided us with more insight on the differences in sensitivity between the two species. With these findings it is now possible to interpolate LLNA dose response data towards an estimation of risk in man. At this point, the GPMT and the LLNA are used for the identification of sensitizing chemicals leading to labelling. If it is possible to determine thresholds in humans, for certain very weak allergens this may ultimately lead to the descision not to label a chemical as an allergen. The use of cytokines for the assessment of sensitizing potency seems promising and is one of numerous possibilities of *in vitro* tests for the assessment of sensitizing potency. Currently, the application of genomic technology to toxicology (toxicogenomics) is a relatively new and rapidly developing discipline holding great

promise. Hazard identification and risk assessment processes will advance from such genomics techniques, which will definitely lead to greater understanding of action of allergens, development of novel biomarkers of exposure and effect, and better identification of sensitive subpopulations.

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SAMENVATTING EN DISCUSSIE

De huid fungeert niet alleen als een fysisch-mechanische barrière tussen het lichaam en zijn/haar omgeving, maar treedt ook op als een actief immunologisch orgaan. Allergische aandoeningen van de huid zijn vaak heel vervelend voor de persoon in kwestie en vormen een serieus probleem. Een van de belangrijkste en meest voorkomende huidaandoeningen veroorzaakt door chemische stoffen is contact overgevoeligheid (CHS), ook wel bekend als sensibilisering van de huid. Door een veranderende levensstijl en een toename van het aantal chemische stoffen waaraan men blootgesteld wordt, groeit de laatste jaren de prevalentie van CHS proportioneel. Daarom wordt er op grote schaal gebruik gemaakt van voorspellende diermodellen om stoffen die een allergische reactie in de huid kunnen veroorzaken tijdig te identificeren. Naast identificatie, is het voor een goede inschatting van het risico ook noodzakelijk om de mate van sensibiliserende potentie (kwantificering) vast te stellen. Deze ontwikkelingen resulteren in een toename in het gebruik van proefdieren. Een doelstelling van het onderzoek zoals beschreven in dit proefschrift was om het proces van risicoschatting te evalueren op een betrouwbare manier. Een tweede doelstelling was om de mogelijkheid te onderzoeken een *in vitro* model te ontwikkelen voor de identificatie van huidallergenen. Beide doelstellingen passen in het streven naar een verfijning, vermindering of vervanging van bestaande diermodellen (3 V's).

De meest gehanteerde diermodellen voor de identificatie van huidallergenen zijn de "guinea pig maximization test" (GPMT) in de cavia en de "local lymph node assay" (LLNA; lokale lymfklier test) in de muis. De LLNA meet de proliferatie van de cellen in de lymfeklieren die de plaats van blootstelling (oortjes) draineren, als een maat voor de allergene potentie van een stof. **Hoofdstuk 2** beschrijft een studie waarbij de allergene potentie van 10 bekende allergenen is bepaald met behulp van een dosis-respons analyse in de LLNA. Doorgaans wordt een stimulatie-index (SI) van 3 gehanteerd als "cut-off" in de standaard LLNA, waarbij stoffen die boven deze grens uitkomen als allergeen worden bestempeld. Onze methode, daarentegen, beschouwt alle datapunten (dieren) die bijdragen aan de dosis-respons curve om zo de EC_3 (effectieve concentratie die een 3-voudige toename van label in het DNA van de cellen induceert) waarde te bepalen. Daarnaast verschaft deze methode ook informatie over de betrouwbaarheid van de EC_3 waarde door de toepassing van de zgn. "bootstrap approach". Deze methodiek levert onzekerheids marges wat belangrijk is voor de onderlinge vergelijking van allergenen. De methodiek gehanteerd in deze studie is een meer nauwkeurige manier (met informatie over de betrouwbaarheid) om de allergene potentie (lees: sterkte) van sensibiliserende verbindingen te bepalen. Bovendien geeft het een schatting van de laagste concentratie nodig om een allergene respons te veroorzaken (belangrijk voor risico-evaluatie). De bovengenoemde methode is in een vervolgstudie toegepast voor het testen van 15 verbindingen gebruikt in de productie van latex handschoenen (als ontharders, katalysatoren etc.). Na bepaling van de allergene potentie, op basis van de EC_3 waarde van deze verbindingen, werden deze geclassificeerd van sterk naar zwak allergeen (of zelfs niet allergeen). Deze bevindingen staan beschreven in **hoofdstuk 4.** Gebaseerd op de uitkomst van deze studie kunnen nu de verbindingen worden geselecteerd, die minder of zelfs niet allergeen zijn voor de productie van natuurlijke rubber latex producten.

De standaard GPMT hanteert één enkele concentratie voor inductie en verschaft een kwalitatieve bepaling van de allergeniciteit (in tegenstelling tot een kwantitatieve bepaling in de LLNA). De experimentele opzet van de GPMT, waarbij de mate van roodheid op de huid van de cavia wordt gehanteerd als maat voor allergeniciteit, is niet geschikt voor de vaststelling van een 'threshold' (=drempelwaarde). Om een onderlinge vergelijking mogelijk te maken hebben we getracht de GPMT op een meer kwantitatieve manier toe te passen door gebruik te maken

van meerdere concentraties in de inductiefase (dosis-respons; **hoofdstuk 3**). Hierbij werd zowel het aantal positieve dieren als de mate van huidreactie gebruikt voor de evaluatie van de allergene potentie van 3 model-allergenen. Dit resulteerde in een verschillende ranking van de betreffende allergenen in de GPMT en de LLNA. Hoewel de semi-kwantitatieve interpretatie in de GPMT de dosis-respons modellering bemoeilijkt, lijkt deze test gevoeliger te zijn dan de LLNA. Bovendien meet de GPMT responsen in de challenge fase (de 'echte' respons), wat een betere reflectie geeft van de werkelijkheid vergeleken met de LLNA welke meet in de sensibilisatie fase.

Hoe vaak iemand blootgesteld wordt (frequentie) zou mede bepalend kunnen zijn voor de uiteindelijke uitkomst van een immunologische respons. In de praktijk wordt men vaak maanden of jaren blootgesteld, terwijl de duur van blootstelling in de LLNA 'maar' 3 dagen is. Wij hebben getracht de invloed van langdurig blootstellen te onderzoeken met de LLNA. De dieren zijn daarbij 2 maanden blootgesteld aan concentraties lager dan de EC_3, om te kijken of de proliferatieve responsen uiteindelijk boven een SI=3 uitkwamen (beschreven in **hoofdstuk 5**). Daar dit niet het geval was kunnen we concluderen dat de kortdurende blootstelling in de LLNA voldoende is voor identificatie en kwantificering van allergenen.

De LLNA is verder ontwikkeld om huid (contact) allergenen en respiratoire allergenen van elkaar te onderscheiden. Hierbij wordt uitgegaan van de aanname dat contact allergenen een Th1 respons (zoals IFN-γ productie) en respiratoire allergenen een selectieve Th2 respons (zoals IL-4 productie) induceren. Deze methode vergelijkt cytokinen profielen op basis van één enkele concentratie, waarbij de vergelijking zou moeten berusten op basis van gelijke SI's en dus afhankelijk is van de concentratie van het allergeen. Daarom hebben wij de mogelijkheid onderzocht om deze twee typen allergenen van elkaar te onderscheiden op basis van dosis-afhankelijke cytokinen profielen (**hoofdstuk 6**). Wij vonden dat, naast de duidelijke IFN-γ productie door contact allergenen en IL-4/IL-10 productie door respiratoire allergenen, ook allergenen van een gemengde aard bestaan, waarbij het profiel afhankelijk is van de concentratie. Deze bevinding onderschrijft nog eens hoe belangrijk het is om bij deze type studies een concentratiereeks te gebruiken in plaats van één enkele concentratie. De methode, zoals wij deze hebben toegepast, is een enkele korte assay die zowel de proliferatie (allergene potentie) als de cytokine-productie (onderscheid tussen contact- en respiratoire allergenen) bestudeert en lijkt daarom een betere methode en is bovendien in lijn met het beleid over het welzijn van dieren ("animal welfare policies").

Een alternatief voor *in vivo* testen, voor identificatie van sensibiliserende capaciteit, is de ontwikkeling van experimentele mathematische modellen. Allergenen hebben specifieke eigenschappen waardoor ze in staat zijn een allergische respons te veroorzaken. Voor laag-moleculaire allergenen zijn belangrijke parameters o.a. reactiviteit en lipofiliciteit, waarbij correlatie tussen deze eigenschappen en sensibilisering van de huid reeds is aangetoond. Gebaseerd op deze gegevens hebben we getracht een mathematisch model te ontwikkelen, waarin een aantal moleculaire kenmerken belangrijk voor permeabiliteit door de huid en reactiviteit in de huid zijn opgenomen, om de allergene potentie van stoffen te voorspellen (**hoofdstuk 7**). Regressie-analyse van de EC_3 met de verschillende parameters/eigenschappen heeft geresulteerd in verschillende mathematische vergelijkingen waaruit "predicted sensitizing values" (PSV's) kunnen worden berekend. Na classificatie van de verschillende allergenen konden de relatief zwakke allergenen van de sterkere worden onderscheiden.

Epidermale keratinocyten (KC) produceren een verscheidenheid aan pro-inflammatoire cytokinen, chemokinen en groeifactoren. Er is reeds aangetoond dat zij een rol spelen in een aantal immunologisch gemediëerde huidziekten. Bovendien zijn ze makkelijk te kweken, wat van deze cel een geliefd instrument maakt voor *in vitro* testen. Relatieve allergene potentie is onderzocht door cytokinen dosis-respons relaties in een muize KC cellijn HEL30 (**hoofdstuk**

8) en een humane KC cellijn HaCaT (**hoofdstuk 9**) vast te stellen. Deze werden vervolgens geanalyseerd, waarna EC_3 waarden zijn berekend. Vervolgens zijn deze gebruikt voor classificatie van de verschillende allergenen. De ranking zoals wij deze vonden in de muis (HEL30) kwam overeen met de classificatie zoals wij deze reeds vonden *in vivo* (LLNA). Bovendien zagen we dat de classificatie *in vitro* in beide species sterk overeen kwamen.

De bovengenoemde *in silico* (**hoofdstuk 7**) en *in vitro* (**hoofdstuk 8** en **9**) benaderingen kunnen worden gezien als mogelijke methoden om de allergene potentie van stoffen te voorspellen, die mogelijk zullen leiden tot een vermindering van het proefdiergebruik. Desalniettemin is validatie van deze methoden noodzakelijk (testen van meerdere allergenen, niet-allergenen en irriterende verbindingen). Over het algemeen zijn de afwezigheid van biotransformatie in gekweekte cellen, mogelijk niet-specifieke immunologische effecten van teststoffen en de afwezigheid van externe invloeden (zoals andere celtypen of neuro-endocrine interacties) belangrijke tekortkomingen van *in vitro* methoden. Daarentegen hebben *in vitro* assays ook een aantal belangrijke voordelen vergeleken met *in vivo* assays: ze besparen dieren (en daardoor) geld en tijd. Bovendien kan een bredere concentratie-range worden bekeken.

Met de benadering zoals deze beschreven staat in dit proefschrift, zijn we nu in staat om de allergene potentie van sensibiliserende verbindingen in de LLNA op een nauwkeurig(er) en betrouwbaar(der) vast te stellen. Dit resulteert in een betere inschatting van het minimale aantal proefdieren nodig voor een experiment, wat uiteindelijk zal leiden tot een vermindering van het proefdiergebruik. Daarnaast verschaft deze predictieve methode ons cruciale informatie over de (mogelijke) threshold van een allergeen. Het 90%-betrouwbaarheidsinterval geeft informatie over de kwaliteit van een bepaalde dataset, waarbij de onderste 5%-grens voorspelt dat onder de betreffende concentratie (EC_3) er een 5% kans bestaat op een positieve respons (sensibilisatie) en kan daarom worden gezien als een threshold. Thresholds in mensen kunnen niet direct worden afgeleid van dierexperimenten. Daarom hebben we getracht een *in vitro* model te ontwikkelen waarbij de productie van cytokinen door muize- en humane huidcellen (keratinocyten) als indicatie voor allergene potentie is gebruikt. Deze *in vitro* benadering verschaft ons enig inzicht in de onderlinge verschillen in gevoeligheid tussen mens en muis, belangrijk voor de vertaling van LLNA data (muis) naar een risicoschatting in de mens. De GPMT en de LLNA worden gebruikt voor de identificatie van sensibilisrende stoffen wat leidt tot labelling. Als het mogelijk is om thresholds bij mensen vast te stellen, zal dit uiteindelijk leiden tot een beslissing om sommige zwakke allergenen niet te labelen als zijnde allergeen. Cytokinen als “tool” voor de vaststelling van allergene potentie lijken veelbelovend en is één van de vele mogelijke *in vitro* benaderingen. Momenteel is een relatief nieuw en dynamische technologie binnen de toxicologie de “toxicogenomics”, waarvan veel wordt verwacht in de toekomst. Risico-identificatie en -schatting zullen door de toepassing van dit type technieken worden verbeterd, wat onherroepelijk zal leiden tot meer inzicht in de werking van allergenen, ontwikkeling van nieuwe biomarkers voor blootstelling/effect en identificatie van gevoelige subpopulaties.

ABBREVATIONS

ANOVA	analysis of variance
BENZ	benzocaine
BSA	bovine serum albumin
CHS	contact hypersensitivity
DEA	diethylamine
DMEM	Dulbecco's modified eagle's medium
DNCB	dinitrochlorobenzene
DTH	delayed-type hypersensitivity
EC_3	estimated concentration in % required for SI=3
ELISA	enzyme-linked immunosorbent assay
FCS	foetal calf serum
HaCaT	human keratinocyte cell line
HEL30	murine keratinocyte cell line
HCA	hexylcinnamic aldehyde
GPMT	guinea pig maximization test
ICAM	intercellular adhesion molecule
ICE	IL-1β coverting enzyme
IL	interleukin
KC	keratinocytes
LC	Langerhans cells
LLNA	local lymph node assay
LN	lymph node
MBT	mercaptobenzothiazole
MBTS	dibenzothiazyldisulfide
MHC	major histocompatibility complex
min	minutes
n	number
NHEK	normal human epidermal keratinocytes
NK	natural killer
OXA	oxazolone
PA	phtalic anhydride
PROAST	possible risk obtained from animal studies
PSV	predicted sensitizing value
PTD	dipentamethylenethiuramdisulfide
PTT	dipentamethylenethiuramtetrasulfide
QSAR	quantitative structure-activity relationship
SI	stimulation index
TBTD	tetrabutylthiuramdisulfide
TDI	toluene diisocyanate
TETD	tetraethylthiuramdisulfide
Th	T helper
TMA	trimellitic anhydride
TMTD	tetramethylthiuramdisulfide
TMTM	tetramethylthiurammonosulfide
TS_5	estimated concentration in % required for a total skin score of 5
ZDBC	zinc dibutyldithiocarbamate
ZDEC	zinc diethyldithiocarbamate
ZDMC	zinc dimethyldithiocarbamate
ZMBT	zinc mercaptobenzothiazole
ZPC	zinc pentamethylenedithiocarbamate

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CURRICULUM VITAE

De auteur van dit proefschrift werd op 3 april 1971 geboren te Eindhoven. In 1991 behaalde hij het VWO-diploma aan het Sint-Joris college te Eindhoven, na in 1989 reeds het HAVO-diploma te hebben behaald. In het daaropvolgende jaar werd de dienstplicht vervuld waarin hij als ambulance-chauffeur werkzaam was op de Willem George Frederik kazerne te Harderwijk. In 1992 begon hij met de studie Biologie aan de Universiteit Utrecht (UU). Tijdens deze studie liep hij stages bij de vakgroep Moleculaire Biochemische Toxicologie op het toenmalige Research Instituut voor Toxicologie (nu Institute for Risk Assessment Sciences) o.l.v. Dr. W.A. Kappers en Dr. G.J. Horbach, en de vakgroep Pathologie in het Academisch Ziekenhuis Utrecht (nu Universitair Medisch Centrum Utrecht) o.l.v. Dr. P. Joling en Dr. R.A. de Weger. Na het behalen van het doctoraal examen in januari 1998, begon hij in februari 1998 aan zijn promotiestudie bij de vakgroep Pathologie aan de Faculteit Diergeneeskunde van de UU in samenwerking met het Laboratorium Pathologie en Immunobiologie van het Rijksinstituut voor Volksgezondheid en Milieu (RIVM) te Bilthoven. Het promotieonderzoek, waarvan de resultaten beschreven staan in dit proefschrift, is uitgevoerd onder begeleiding van Dr. R.J. Vandebriel, Prof. Dr. H. van Loveren en Prof. Dr. J. Vos.

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